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Monosynaptic Excitation of Alpha Motoneurones from Supraspinal Structures in the Cat

By

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Abstract

LUND, S and O POMPEIANO. *M nosynaptic xcit tion f alpha motoneurones from supraspi al structures n th at* Acta physiol. scand. 1968 73 1—21

The origin of descending fibres evoking monosynaptic excitatory postsynaptic potentials (EPSPs) in motoneurones was studied. Lumbar flexor and extensor motoneurones were recorded from intracellularly The ipsilateral ventral and lateral funicles were stimulated at thoracic le 1 in cats with and without chronic lesions of the ipsilateral cervical cord or vestibular complex. I other experiments the ipsilateral Deiters nucleus was stimulated. Volley in descending ipsilateral fibres evoke monosynaptic EPSP in the majority of the flexor and extensor motoneurones with an amplitude corresponding to about 5 pe cent of the homonymous group Ia EPSP The effect is almost entirely of supraspinal origin. Most extensor motoneurones recei this action from fibres originating in the ipsilateral Deiters nucleus. Flexor and probably some species of extensor motoneurones, recei the action from supraspinal structure outside the ipsilateral vestibular complex. The results are discussed in relation to the cerebellar influence on the Del ers nucleus and the cerebellar inhibition of extensor rigidity

It has been assumed that th only descending pathway which exerts a monosynaptic nfluence on alpha motoneurones is the pyramidal tract (Bernhard and Bohm 1954a, b, Phill ps and Porter 1964) This connexion is present in the primates but not in the cat (Lundberg and Voorhoeve 1962) where this pathway acts on the motoneurones through interneurones of the reflex pathways. However Lloyd (1941) de scribed a descending pathway in the ventral quadrant of the spinal cord, which ends directly upon the alpha motoneurones of the lumbosacral cord and facilitates the monosynaptic reflex. This pathway was called the bulbospinal correlation system, ince t was show to originate from brain stem neurones and from propriospinal neurones. Since Lloyd' original observation the suggestion has been put forward that this pathway may originate either from the reticular formation (Magoun 1950, Sasaki, Tanaka and Mori 1962) or from the vestibular nuclei (Gernandt, Katsuki and Livingston 1957 *cf* Gernandt and Gilman 1960a, b) However recent ana tomical tudies on the mode of termination of th reticulospinal (Nyberg *Hansen*

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1965) and vestibulospinal tracts (Nyberg-Hansen 1964, Nyberg-Hansen and Mascitti 1964) does not indicate that these pathways make any substantial monosynaptic contact with motoneurones.

The method of intracellular recording is particularly suitable for investigating the question whether brain stem neurones are connected monosynaptically with alpha motoneurones in the spinal cord. Recent experiments with this technique have shown that lumbar motoneurones in the cat receive monosynaptic excitation from impulses in axons descending in the ipsilateral ventral quadrant (Eide, Lundberg and Voorhoeve 1961).

The aim of the present experiments was to study the pattern of monosynaptic excitatory impingement in alpha motoneurones by volleys in descending fibres and to establish the origin of these pathways. Preliminary reports have been given (Lund and Pompeiano 1965, Pompeiano 1966).

Methods

The experiments were performed in 18 adult cats submitted under ether anaesthesia to precollicular decerebration or anaemic decortication (Andén *et al.* 1966). The right sciatic and hamstring nerves and the obturator and quadriceps nerves on both sides were cut, and the following nerves were dissected on the left side: PBSt, ABSm, G-S, FDL, Pl, Tib, DP (abbreviations explained below).

Lumbar motoneurones were recorded intracellularly with microelectrodes filled with 3 M KCl or 2 M K-citrate. Superposition of many faint traces was employed. The dorsal and the ventral roots were generally left intact. In this way (1) the cells recorded from were identified as motoneurones when they were invaded antidromically by stimulation of muscle nerves, (2) it was possible to compare the EPSP induced by descending volleys with the EPSP induced by stimulation of the primary afferents. Occasionally the ventral roots L7 and S1 were cut and mounted for stimulation. In those cases the motoneurones were identified by recording their ... receptiveness (Eccles, Eccles and Lundberg 1957).

Descending volleys and ... in the dorsal root were recorded from the cord dorsum by ... tipped ... electrode placed near the entry point of the microelectrode against an indifferent electrode in the muscle. The stimulus pulses used were condenser discharges with a half decay of 45 µsec.

Three different series of experiments were performed. In the first series (10 cats) the animals were spinalized between T10 and T12 and the responses of lumbosacral motoneurones to spinal cord stimulation were investigated. The dorsal columns were removed for about one segment below the transection. In 5 animals the ipsilateral ventral and lateral funicles were dissected together and placed on stimulating electrodes. In 4 experiments the ipsilateral ventral and lateral funicles were split in two parts (one including the ventral funicle and ventral part of lateral funicle, the second including the dorsal part of lateral funicle) each one being used for separate stimulation. In the last experiment the ipsilateral ventral and lateral funicles were stimulated ... postbrachial level 27 days after ... almost complete ipsilateral hemisection of the spinal cord (C3–C4) which spared the medial part of the ventral funicle.

In a second series of experiments 3 ... the ipsilateral Deiters nucleus was stimulated. Correct placing of the electrode was guided by the Horsley-Clark co-ordinates and by checking the increase in extensor tonus induced in the ipsilateral forelimb by repetitive stimulation before the animal was ... Because of the ... the response of the ipsilateral hindlimb could not be seen. After the experiments an electrolytic lesion was performed to permit histological control.

In a third series of experiments 5 ... the animals were submitted under Nembutal anaesthesia to partial or complete stereotaxic electrolytic lesions in the vestibular nuclei on one side. 1 —33 days after the lesion, i.e. following degeneration of the efferent pathways from the vestibular complex, the animals were submitted under ether anaesthesia to the same kind of operation as those described in the first series of experiments. In these experiments also the contralateral ventral and lateral funicles were stimulated together at T10—11 level.

The data reported here have been derived from intracellular records from 715 moto-

neurones, 306 motoneurones belonged to the first, 187 to the second and 222 to the third series of experiments. The recording started 1—2 hours after the ether anaesthesia was discontinued. The animals were curarized with gallamine triethiodide (Flaxedil, May and Baker Ltd.) and artificially respired with a mixture of 6 per cent CO_2 in O_2. The blood pressure was recorded continuously during the experiment.

The anatomical localization of the stimulating electrode in Deiters' nucleus, of the chronic lesions in the vestibular nuclei and in the cervical cord was controlled at the end of the experiment in serial histological sections stained with Nissl technique. The recent delimitation of the vestibular nuclei was adopted (Brodal and Pompeiano 1957). When the ventral and lateral funicles were split into two parts for separate stimulation this subdivision was also controlled with the same histological technique.

The following abbreviations are used: excitatory postsynaptic potential, EPSP; inhibitory postsynaptic potential, IPSP; ipsilateral, i; contralateral, c; posterior biceps-semitendinosus, PBSt; anterior biceps-semimembranosus, ABSm; gastrocnemius-soleus, G-S; flexor digitorum and hallucis longus, FDL; plantaris, Pl; tibial, Tib; deep peroneal (tibialis anterior and extensor digitorum longus), DP; ventral and lateral funicles, VLF; lateral vestibular nucleus (Deiters), LVN.

Results

1 *Descending volleys elicited by stimulation of the ventral and lateral funicles*

Single shock stimulation of the ipsilateral ventral and lateral funicles (iVLF) at T10—T12 produced an early and a late descending volley which could be recorded as positive potentials from the dorsal surface of the spinal cord at L7—S1 (Fig. 1). Measurement from the dorsolateral white matter cannot be used to state the exact time of arrival of descending impulses at their synaptic terminals, because the tapering arborization and level of entry into the grey matter of the descending fibres is not precisely known. However, the peak of the early positive wave indicates the

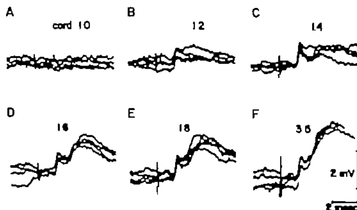


Fig. 1. Mono- and polysynaptic EPSPs evoked in a motoneurone by an increasing descending volley. Intracellular records from an ABSm motoneurone. In this and following figures upper traces are intracellular records, internal positivity (depolarization) being signalled upwards. Lower traces are recorded between a surface electrode at the lower L7 or the upper S1 dorsal root entry zone and an indifferent electrode in the back muscles (negativity of the central electrode being signalled upwards). The ipsilateral ventral and lateral funicles (icord) are stimulated together at lower thoracic level. Figures indicate multiples of threshold stimulus strength for the early descending volley. In this and following figures the voltage scales refer only to the microelectrode records.

arrival of a synchronous descending volley. Over an average distance of 10.6 cm between stimulating and recording electrodes, the peak of the early positive wave occurred on average at 0.98 msec latency (mean conduction velocity 108 m/sec) while the peak of the late wave generally followed the peak of the early one at an average interval of 0.90 msec. The early positive potential reached its maximal amplitude at slightly suprathreshold stimulus intensity and higher intensities were required for the late positive potential to reach its maximal amplitude (Fig. 1). It is concluded that stimulation of the ipsilateral VLF at lower thoracic level produces a descending volley conducted to the L_7 segment at about 100 m/sec. These findings are consistent with the observations of Lloyd (1941).

2. *Monosynaptic EPSPs in extensor and flexor motoneurones induced by stimulation of the ventral and lateral funicles*

Single shock stimulation of iVLF evoked EPSPs in flexor and extensor motoneurones (Table I and II, Fig. 1 and 2). To determine the monosynaptic or polysynaptic nature of these EPSPs the following criteria were used. The interval between the peak of the early positive wave and the onset of the EPSP in the motoneurone (the segmental latency) can be precisely measured and is 0.60 msec in Fig. 1. Fig. 3A

TABLE I. Percentage of motoneurones with monosynaptic EPSPs on stimulation of the ipsilateral ventral and lateral funicles (iVLF) at T 10–12 and ipsilateral Deiters nucleus (iND). Total number of motoneurones investigated in parentheses

Structure stimulated	Monosynaptic EPSPs in			
	Extensor motoneurones		Flexor motoneurones	
iVLF	83	(77)	2	(10)
iND 3 ...	75	(12)	15	(42)

TABLE II. Percentage of motoneurones in which a monosynaptic EPSP was evoked on stimulation of the ipsilateral ventral and lateral funicles together at T 10–12 level. Total number of motoneurones investigated in parentheses. See also Table I

Extensor motoneurones			Flexor motoneurones		
G-S	80	(41)	PBSt	72	(60)
Pl	67	(3)	DP		(10)
Tb	75	(1)			
FDL	100	(4)			
ABSm	80	(10)			
Total	83	(7)		72	(60)

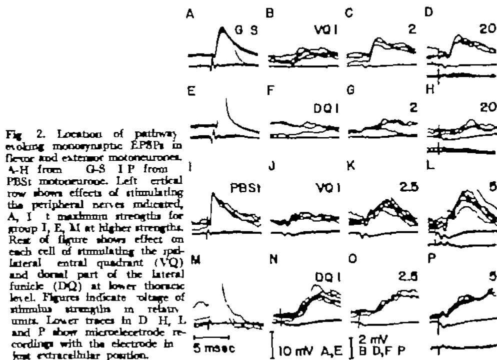


Fig 2. Location of pathway evoking monosynaptic EPSPs in flexor and extensor motoneurones. A-H from G-S I P from PBSt motoneurone. Left ertical row shows effects of stimulating the peripheral nerves indicated, A, I t maximum strengths for group I, E, M at higher strengths. Rest of figure shows effect on each cell of stimulating the ipsilateral entral quadrant (VQ) and dorsal part of the lateral funicle (DQ) at lower thoracic level. Figures indicate oltage of stimulus strengths in relativ units. Lower traces in D H, L and P show microelectrode recordings with the electrode in just extracellular position.

shows the distribution of the delay of early and lat EPSPs n a number of motoneurones. It appears that the segmental latency of the early EPSPs ranges from 0.35 to 0.75 msec, thus being monosynaptic in nature (*cf* Eccles 1964 p. 38) In the different motor nuclei tested bout the same percentage of motoneurones received this monosynaptic EPSP (Table II) DP motoneurones were not tested but evidence from experiments in the third series indicates that also these motoneurones receive descending monosynaptic EPSPs of the same characteristics.

By increasing th stimulus intensity applied to th VLF the monosynaptic EPSP grew progressively until t reached maximal amplitude t a stimulus intensity 1.6—1.8 times its threshold (Fig 1) Whil in most instances a correlation could be found between th development of the rly descending olley and the growth in amplitude of the corresponding monos naptic EPSP in other instances th maximum development of the descend ng monosynaptic EPSP could be reached abruptly *i.e.* at a stimulus ntensity just supraliminal for eliciting the early descending EPSP In these cases no further ncrease in mplitude of this EPSP occurred with the progressi enhancement of the early descending olley This finding i dicates that in some motoneurones th monosynapti EPSP originates from selecti stimulation of a restricted number of f bres of similar threshold, and therefore of simila conduction elocity

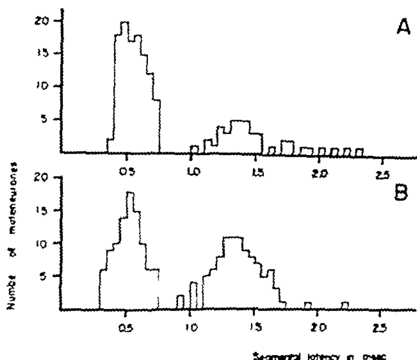


Fig. 3. Histograms of the segmental latency distribution for the mono- and polysynaptic EPSPs evoked in motoneurones by volleys in descending fibres. A, the distribution of segmental latency for the mono- and polysynaptic EPSP in 110 motoneurones (64 extensor and 46 flexor motoneurones) on stimulation of the VLF at lower thoracic level. Only in 45 out of these 110 neurones was the latency of the polysynaptic EPSP clearly measurable. B, the same ... extensor motoneurones on stimulation of the ipsilateral Deiters' nucleus. The latency of the monosynaptic EPSP plotted from 94 out of 125 cells, for the polysynaptic from 93 out of ... cells.

In order to have comparable criteria of the effectiveness of this monosynaptic impingement on motoneurones the maximum amplitude of the monosynaptic EPSP induced by single shock stimulation of the spinal cord should be compared with the maximum amplitude of the monosynaptic EPSP induced by stimulation of the group Ia afferents in the homonymous nerve. There was usually a clear threshold separation between the group Ia fibres and the motor axon. This fact allowed the amplitude of the homonymous monosynaptic EPSP to be determined without the ... antidromic ... In a few exceptional cases, however, the antidromic ... occurred before the group Ia EPSP had reached its maximum amplitude ... by passing hyperpolarizing current through the ... to block the antidromic invasion, as suggested by *Eccles et al.* 19... had ... performed.

The present series has included many ... potentials considerably below the ... of − ... mV, ... attributed ... damaged by the impalement (*Coombs, Eccles and Fatt* 1955a). These low ... potentials ... from damage by the microelectrode and it may be assumed ... the EPSPs of these motoneurones are also proportionally diminished (*Coombs, Eccles and Fatt* 1955...). ... the relative size of the EPSPs set up by the ... should not be affected ...

The average amplitude of the maximum monosynaptic EPSP induced by descending volleys in extensor motoneurones corresponded to 24±1 per cent of the maximum amplitude of the monosynaptic homonymous group Ia EPSP. There was no significant difference in this figure between motoneurones belonging to different motor nuclei. The corresponding value for PBSt motoneurones was 23±16 per cent.

On increasing the stimulus intensity of the iVLF a late EPSP appeared in both flexor and extensor motoneurones with a segmental latency of 1.4 msec, thus indicating a disynaptic linkage (Fig. 1). While the maximum descending monosynaptic EPSP is generally subthreshold for firing, the late EPSP can reach the firing threshold. As it could be expected intravenous injection of small doses (3 mg/kg) of Nembutal decreased or abolished this late EPSP, while the monosynaptic EPSP was unmodified or only slightly reduced in amplitude.

The following experiments were performed in order to find out in which part of the iVLF the descending pathways evoking monosynaptic EPSPs in flexor and extensor motoneurones are located. The dorsal half of the ipsilateral lateral funicle was dissected and placed on one pair of stimulating electrodes, the ventral half of the lateral funicle and ventral funicle on another. A monosynaptic EPSP similar in amplitude to that described on stimulation of the intact VLF was recorded in both extensor (G-S, Pl, Tib, ABSm tested) and flexor (PBSt tested) motoneurones, when the ventral half of the lateral funicle and ventral funicle were stimulated, but the effect was only occasionally evoked from the dorsal half of the lateral funicle (Fig. 2, Table III, upper two entries). Since the splitting of the VLF is likely to cause damage of fibres, the results in Table III (upper two entries) have not been included in the results in Table I (upper entry). This is of importance when judging to what extent the descending pathways evoking monosynaptic EPSPs in motoneurones are

Table III. Percentage of motoneurones with monosynaptic EPSP on stimulation of different parts of the ipsilateral ventral and lateral funicles (iVLF) at Th13–L1 in intact cats (4 cats) and in one animal 7 days after a chronic spinal cord lesion. Total number of motoneurones investigated in parentheses

Structure stimulated	Monosynaptic EPSPs in			
	Extensor motoneurones		Flexor motoneurones	
Dorsal half of lateral funicle	0	63	4	[illegible]
Ventral half of lateral funicle and ventral funicle	46	[illegible]	5	46
VLF after ipsilateral hemisection at C_{3-4} sparing medial part of ventral funicle	44	[illegible]	3	15

of supraspinal origin. It is concluded from the results of these experiments that the pathways with this action on the flexor and extensor motoneurones tested are both located in the ventral quadrant of the spinal cord.

According to Willis *et al.* (1967) monosynaptic EPSPs can be evoked in motoneurones by fibres running in both the ipsilateral and contralateral ventral quadrant. In our material, however, stimulation of the contralateral VLF produced an EPSP with a minimum segmental latency of 1.2—1.5 msec (Fig. 9 and 10) and an amplitude on the average 2.3 times larger than the corresponding monosynaptic EPSP induced by stimulating the ipsilateral VLF. The contralateral volley sometimes evokes large extracellular field potentials and an accurate measurement of the segmental latency can only be made if extracellular fields are taken (Fig. 9). The difference in latency between early EPSPs evoked from the ipsi- and contralateral sides is particularly well illustrated in Fig. 10 where ipsi- and contralateral traces are superimposed in D. It is postulated that the contralateral early EPSP is a disynaptic effect.

In order to find to what extent the descending fibres evoking monosynaptic EPSPs in motoneurones are of supraspinal origin the following experiment was performed. The responses of motoneurones to stimulation of the iVLF were investigated 27 days after an almost complete, ipsilateral hemisection of the spinal cord at C3—C4 which spa[illegible]ed *the medial part of the ventral funicle (Fig. 4G). Monosynaptic effects* were evoked in extensor (G-S, FDL, ABSm tested) but absent in the majority of the flexor (PBSt and DP tested) motoneurones (Table III, lower entry, Fig. 4). This finding strongl[illegible] suggests that the pathway with monosynaptic connexion to [illegible]r motoneurones is of entirely supraspinal origin, b[illegible]t for the effect to extensor [illegible]eurones no conclusions can be drawn. Evidence of a different kind will, how[illegible]er be presented below showing that the effect is almost entirely of supraspinal [illegible]igin f[illegible]r extensor mo[illegible]neurones also. Taking this into account the present experiment now [illegible]uggests that th[illegible] descending pathways with monosynaptic action to flexor and exten[illegible]or [illegible] neurones are differently located at the cervical level.

The prese[illegible]t experiment provid[illegible] onl[illegible] a few data bearing on the question of differenti[illegible]l distrib[illegible]tion [illegible] the [illegible]apses on somatic and dendritic motoneuronal membrane for descend[illegible]g f[illegible]bres and group Ia fibres (cf. Fadiga and Brookhart 1960). The tim[illegible] co[illegible]rse [illegible] th[illegible] mono[illegible]naptic EPSP evoked by volleys in descending fibres generally correspond[illegible] t[illegible] th[illegible] time co[illegible]rse of the monosynaptic group Ia EPSP. In most cases the rise t[illegible] [illegible] th[illegible] response t[illegible] [illegible]pinal cord stimulation wa[illegible] a[illegible] fast a[illegible] that resulting from a l[illegible] [illegible] p Ia [illegible]lley (Fig. 2A–D). In some cases, however a sl[illegible]ghtly longer rise t[illegible] [illegible] th[illegible] [illegible]escend[illegible]ng EPSP could be detected. The decay of the mono[illegible]naptic EPSP w[illegible] [illegible] [illegible] th[illegible]t induced by group Ia st[illegible]mulation.

Analysis of the dur[illegible] [illegible] [illegible]ter h[illegible]perpolarization following the antidromic [illegible]pike (Eccles *et al.* 19[illegible] [illegible] [illegible] that *α* motoneurones bot[illegible] to slow and fa[illegible]t muscles receive mono[illegible]napt[illegible] [illegible] from descend[illegible]ng [illegible]olleys. However these two la[illegible]t problems need further exp[illegible]ental data and will be dealt with in more detail in forthcoming papers (Grillner, H[illegible]ngo and L[illegible]nd, to be published).

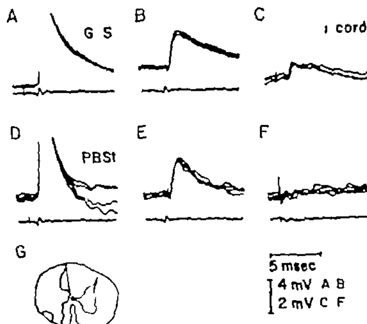


Fig. 4 Effects of chronic lesion at cervical level on monosynaptic EPSPs in motoneurones by descending volleys. A-C from a G-S D-F from PBSt motoneurone. The peripheral nerves indicated ar stimulated in A, D t strengths abov threshold for motor von, in B, E at maximum for group I. C, F show effects of stimulating VLF (lcord) at lower thoracic lev l. G show extension of the ipsilateral chronic lesion t C3—C4 level made 27 day before the acut experiment.

3 M nosynapt EPSP in mot neurones nduced by st mulation of the Deit s ucleus

Single shock stimulation with an electrode placed in the ipsilateral Deiters nucleus produced an early positive potential recorded at the L7 dorsal root entry zone with a latency between the shock artifact and the peak of 2.8 msec. indicating an a erage conduction velocity of 96 m sec. On increasing the stimulus intensity a second positive wa e followed, with a alue for the corresponding latency of 3 5 msec (Fig 6B) Single shock stimulation generated short latency EPSPs in the majority of extensor motoneurones occurring at an average segmental latency of 0.50 msec and are hence monosynaptically evoked. Fig 3B shows the distribution of the segmental latency for mono- and polysynaptically evoked EPSPs by descending volleys in a number of extensor motoneurones. Note the lack of observations at a segmental latency of 0.7—1 0 msec. Fig 5 shows the EPSPs set up in a G-S motoneurone by an increasing vestibulospinal volley The early descending EPSP is often followed by a late EPSP w th a segmental latency of 1 4 msec, thus indicating a disynaptic lnae

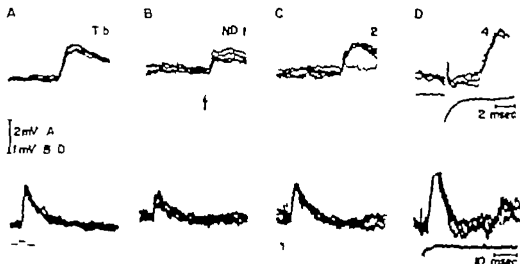


Fig. 5. Mon[o]synaptic EPSP in an extensor motoneurone on stimulation of the ipsilateral Deit[e]rs' nucleus. Records from Tib motoneurone. A show the maximal group a EPSP. B-D show the [e]ffect of increasing stimulation of the ipsilateral Deiters' nucleus (ND) with the [a]rrow in B indicating the peak of the [e]arly descending volley. Figures indicate stimulus [v]oltage ... units. Upper and lower records are identical but obtained [a]t different sweep [s]peed.

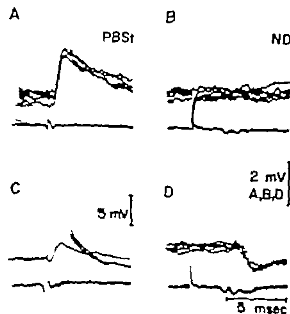


Fig. 6. Effect of stim[u]lation of the De[i]ters' nucleu[s] on [a] flexor motoneurone. A, B from one, C, D from another PBS[t] motoneurone in the same experiment. The nerve to PBSt muscle is stimulated [in] A maximal, in C supramaximal strength for group Ia. In B, D the Deiters' nucleu[s] is stimulated [a]t [a] str[e]ngth supramaximal for the descending [v]olley.

The distribution of the ... [s]ynapt[ic] effect[s] to different mot[o]r nuclei are [s]umma[r]ized in Table IV. The ... [av]er[a]ge a[m]pl[itu]d[e] of this EPSP wa[s] ... per cent of th[e] homonymou[s] gr[o]up Ia EPSP. [Th]er[e] w[as] no [si]gnificant d[i]fference in this figure between m[o]t[o]neurones belon[ging] t[o] diff[e]rent m[o]tor n[u]clei. This [v]alue is similar to

TABLE IV Percentage of motoneurones in which monosynaptic EPSP was evoked on stimulation of the Deiters nucleus. Total number of motoneurones investigated in parentheses. See also Table I

Extensors			Flexors		
G-S	94**	(47)	PBS	21**	(42)
Pl	71	(7)	DP	0	(20)
Tib	69	(42)			
FDL	100	(7)			
ABSm	41	(22)			
Total:	75**	(125)		15**	(62)

that obtained in extensor motoneurones when the ipsilateral ventral and lateral funicles are stimulated at the thoracic level. On the basis of this finding and a comparison of the results in Table II and IV it is postulated that the descending fibres to G-S, Pl, Tib and FDL motoneurones evoking monosynaptic EPSPs are almost entirely of supraspinal origin and a substantial propriospinal contribution can be excluded. For the effect to ABSm motoneurones the possibilities of a propriospinal contribution or a supraspinal source outside the Deiters' nucleus remains open.

In 85 per cent of the flexor motoneurones recorded from, single shock stimulation of the Deiters nucleus did not evoke any monosynaptic EPSP (Fig. 6, Table IV). The effects in flexor motoneurones were mainly inhibitory (Fig. 6D) and often the segmental latency of the IPSP was 1.1—1.3 msec. This latency indicates a disynaptic nature of the response. In a later series of experiments (Grillner, Hongo and Lund, to be published) in which more precise methods have been employed to ascertain that stimulation is limited to the Deiters nucleus, monosynaptic EPSPs were never evoked in flexor motoneurones. Hence it is likely that the effect in flexor motoneurones in Table IV is caused by co-stimulation of a structure outside the ipsilateral Deiters nucleus. The effect to flexor motoneurones from the vestibulospinal tract will be dealt with further in forthcoming papers (Grillner, Hongo and Lund, to be published).

The significance of the monosynaptic EPSP in extensor motoneurones evoked by volleys in the vestibulospinal tract may be deduced from the following findings. It was repeatedly observed that a group Ia EPSP subthreshold for firing, reaches the firing level if conditioned at an appropriate interval by the monosynaptic vestibulospinal EPSP. Changes in the excitability of the soma-dendritic membrane by volleys in the vestibulospinal tract were also tested by activating the cell antidromically. Stimuli were applied at time intervals adequate to prevent the invasion of the dendritic membrane (Eccles 1955). Fig. 7A shows the antidromic motoneurone with frequent failures in the appearance of the SD spike potential. Wh n conditioned ... increasing stimulation of

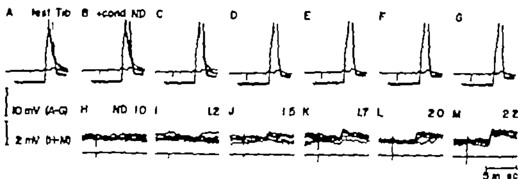


Fig. 7. Deiters facilitation of antidromic invasion. Lower traces in A-G, upper traces in H-M are intracellular records from a Tib motoneurone. A shows the effect of test stimulus to the homonymous nerve at a strength just suprathreshold for the motor axon. B-G show the effects of conditioning with stimulation of Deiters' nucleus with increasing strength. H-M show the effects of conditioning stimulus alone at high gain. Figures indicate stimulus strengths in relative units used for the conditioning stimulus in corresponding upper and lower records.

nucleus in B-G the antidromic invasion is progressively facilitated; the effect of the conditioning stimulation alone is shown at corresponding strengths in H-M.

4. Eff cts of h ont ve t bula le i ns on the monosynapt c EPSP indu ed by stim ulation of the ipsilateral ventral and lateral funicles

In stimulation experiments it may be difficult to exclude effects due to spread of current to adjacent nuclei or to fibres passing close to the stimulating electrode. This complication can be partly eliminated by mapping out the region from which low hold effects can be obtained (Grillner and Lund 1966). However, in the present ment a d fferent approach was used. The effect of stim lation of the iVLF died n 5 cat 1 –33 days after a partial or complete electrolytic lesion of the lateral vestibular nuclei. The extent of some vestibular lesions is shown in Fig. 8. Th result are summarized in Table V. A lesion in ol ing the medial, descending superior nd the forelimb region of the lateral vestibular nuclei did not markedly alter the pattern of descending monosynaptic impingement in those extensor motor n clei tested G-S Pl nd T b. In ABSm mot neurones th pattern wa n ch nged fter a lesi n of the medial and uperior estibular nuclei. However, when the lesion included th nt re De ters nu leus the desc nding olley did not evoke monosynaptic EPSP some mot r nu lei. Fig. 9 illustrates th s for a G-S mot neurone. Of th 31 G-S n ot r rones, onl 3 recei ed monos naptic EPSP from descending olley nd the pl ude w reduced by more than 50 per cent. The effect was completel bolisl l Pl ll tested and Tib (8 ll tested mot neurones, but was still present 7 f th 1 ABSm mot neurones tested (Fig. 10. FDL motoneurones wer not t ed after les on of th uperior descending and lateral estibular nuclei and wer tested n one experiment with a discret lesion of the medial vestibular n clei. Normal effect were ev ked in the motoneurones tested, hence the nucleus the major upr pinal source for descending monosynaptic EPSP in FDL mot neurones.

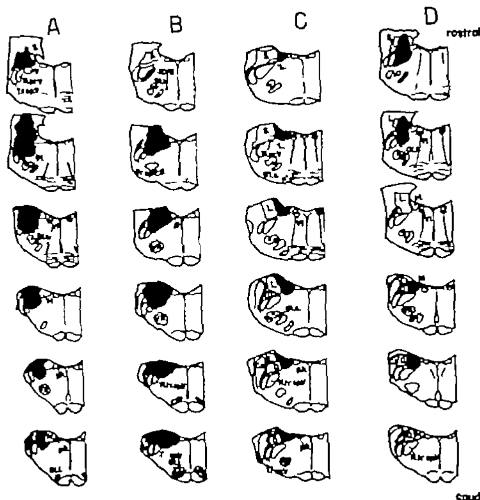


Fig. 6. Unilateral destruction of the vestibular nuclei performed in four different experiments. Schematic representation of brain stem sections, taken at equidistant intervals in the rostro-caudal direction. Shaded areas represent the extent of the electrolytic lesion. A: the lesion affects mainly the superior, lateral and descending vestibular nuclei. B: the lesion affects the lateral, medial and descending vestibular nuclei. C: the lesion is limited to the medial vestibular nucleus. D: the lesion affects the superior and the medial vestibular nucleus as well as the rostral part of the lateral vestibular nucleus, corresponding to the forelimb region of Deiters nucleus (Pompeiano and Brodal 1957). Note the integrity of the caudal part of Deiters nucleus corresponding to the hindlimb region of the nucleus. C.r.: restiform body. D: descending vestibular nucleus. L: lateral vestibular (Deiters') nucleus. M: medial vestibular nucleus. N.pr.V: principal sensory nucleus of trigeminal nerve. N.tr.sp.V: nucleus of spinal tract of trigeminal nerve. N.VII: facial nerve. Ol: inferior olive. Ol.s.: superior olive. p.h.: nucleus praepositus hypoglossi. S: superior vestibular nucleus. Tr.sp.V: spinal tract of trigeminal nerve. V, VI, VII: cranial motor nerve nuclei.

The pathway giving monosynaptic EPSPs in flexor motoneurones to both PBSt and DP motor nuclei remained largely unaffected by the different vestibular lesions. The results indicate that DP motoneurones receive descending monosynaptic EPSP of the same characteristics as the PBSt motoneurones. Fig. 11 shows the

TABLE V. Percentage of motoneurones in which monosynaptic EPSP was evoked on stimulation of the ipsilateral ventral and lateral funicles (iVLF) at T 10—12 12—33 days after lesion of the ipsilateral vestibular nuclei. Total number of motoneurones investigated in parenthesis

Structure stimulated	chronic lesion	Monosynaptic EPSPs in	
		Extensor motoneurones	Flexor motoneurones
iVLF 3 cats	med., sup., desc., forelimb region of lat. vest. nuclei	66 % (135)	100 (6)
iVLF 2 cats	lat. med., sup., desc. vest. nuclei	17 (59)	55 (22)

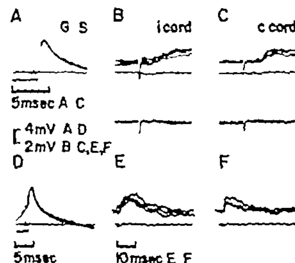


Fig. 9. Abolition of the descending path way evoking monosynaptic EPSP in an extensor motoneurone by a chronic lesion involving the ipsilateral Deiters nucleus. From a G-S motoneurone. A, D show the maximum homonymous monosynaptic EPSP. B, E and C, F show the effects of stimulation of the ipsilateral (i cord) and contralateral VLF (c cord) at lower thoracic level obtained at different sweep speeds. Lower traces in B, C show extracellular fields. The vestibular lesion is illustrated in Fig. 8A.

synaptic EPSP in a PBSt motoneurone evoked from the thoracic spinal cord after a complete lesion of the ipsilateral vestibular nuclei. It is true that in flexor motoneurones only 55 per cent received a monosynaptic EPSP by volleys in the descending fibres (Table V) but this finding cannot be taken to indicate that some flexor motoneurones receive monosynaptic EPSP from the vestibulospinal tract. It is in the nature of the lesion technique that greater emphasis must be placed on the finding that an effect remains after a complete lesion. Further evidence that the descending pathway with monosynaptic action to flexor motoneurones originates from structures outside Deiters nucleus has been given (Grillner and Lund 1966).

To summarize the lesion experiments the results of which are in essential agreement with results reported in the previous section: the ipsilateral Deiters nucleus is shown to be the origin for the descending path way evoking monosynaptic EPSP in

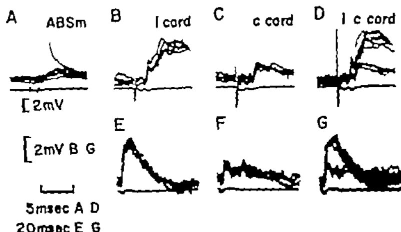


Fig. 10 Persistence of descending pathway evoking monosynaptic EPSP in ABSm motoneurone after lesion involving Deiters' nucleus. A shows the maximal group Ia EPSP B, E how the effects of stimulation of the ipsilateral (Icord) and C, F of the ontrala eral VLF (cord) at T12 I D G the stimulus pulse was switched between the electrodes on the I cord and the cord to show clearly the difference in segmental latencies for the earliest EPSP set p by the tw stimulations. The hronic lesion is illustrated in Fig 8A.

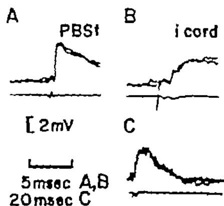


Fig. 11 Persistence of descending pathway evoking monosynaptic EPSP in flexor motoneurone after estibular lesion involving the ipsilateral Deiters nucleus. From PBSt motoneurone A show the maximal group Ia EPSP B, C show the effect of supramaximal stimulation of the VLF at T1 two different sweep speeds The chronic lesion is illustrated in Fig. 8A.

motoneurones belonging to G-S Pl and Tib motor nuclei. For the effect on the ABSm and FDL motoneurones no definite conclusions can be drawn from the present experiments. Forthcoming papers will deal with the origin for th effect in these motor nuclei (Grillner Hongo and Lund, to be published The ipsilateral vestibula complex is not the origin for the effect on flexor motoneurones.

Discussion

The finding that monosynaptic EPSPs are evoked in lumbar motoneurones by descending volleys is well established (Eide *et al.* 1961) According to experiments by Willis *et al.* (1967) in which the cord was stimulated in the L1 segment, these effects are mediated by fibres in both ipsi and contralateral ventral quadrants In

our material. However, only a disynaptic EPSP could be detected in motoneurones following stimulation of the contralateral ventral and lateral funicles and there was never any trace of monosynaptic EPSP. This conclusion is based on the assumption that the fibres mediating the earliest uncrossed and crossed EPSPs have about the same conduction velocity. This apparent discrepancy between the present results and those of Willis *et al.* (1967) may depend on our stimulation having been performed a few segments more rostrally. Hence the effect observed by Willis *et al.* could be caused by activation of a propriospinal pathway taking its origin in the upper lumbar region. The corresponding effects exerted by fibres in the ipsilateral spinal cord, on the other hand, have now been shown to be of supraspinal origin and it can further be concluded that propriospinal pathways originating above the lower thoracic region do not contribute substantially to the descending monosynaptic EPSP. For the effect on G-S, Pl and Tib motoneurones these conclusions are based on the absence of a monosynaptic EPSP on stimulation of the lower thoracic spinal cord after degeneration of the descending pathway caused by a supraspinal lesion. Further evidence is given by the finding that in acute experiments a supraspinal stimulation gives a monosynaptic EPSP in about the same percentage of extensor motoneurones (G-S, Pl, Tib and FDL) and of the same amplitude as that evoked from the ipsilateral ventral and lateral funicles at the lower thoracic level.

The main purpose of the present investigation was to establish the origin of the descending pathway with monosynaptic connexion to motoneurones. This problem has at least been partially solved in that it has been shown that impulses in vestibulospinal fibres originating from Deiters' nucleus, evoke monosynaptic EPSP in an extensor motoneurones. This was demonstrated not only in experiments with electrical stimulation of Deiters' nucleus but also in another series of experiments in which Deiters' nucleus was destroyed by an electrolytic lesion and time allowed for the vestibulospinal fibres to degenerate. Stimulation of the lower thoracic spinal cord was then performed in acute experiments, and in several motor nuclei to extensor muscles no monosynaptic EPSP were evoked when the lesion had produced a complete destruction of Deiters' nucleus. When the results from experiments with lesion and stimulation of the Deiters' nucleus are compiled it appears that G-S, Pl and Tib motoneurones receive all their descending monosynaptic EPSPs entirely from fibres originating from nucleus Deiters. Concerning FDL and ABSm motoneurones it is not possible from the present results to exclude a different supraspinal origin (or, for ABSm motoneurones, that the effect may be evoked partly from propriospinal fibres). Recently Shapovalov (1966) in stimulation experiments concluded that Deiters' nucleus impinges monosynaptically upon motoneurones, but it is not stated whether this effect is exerted on extensor or flexor motoneurones.

By contrast, stimulation of Deiters' nucleus did not usually evoke any monosynaptic EPSP in many motoneurones to flexors. This finding made us suspect that the pathway with monosynaptic connexion to flexor motoneurones takes origin outside Deiters' nucleus. This was confirmed by the lesion experiments: the monosynaptic EPSP in flexor motoneurones could still be evoked from the lower thoracic cord

after degeneration of the vestibulospinal tract following a complete destruction of Deiters nucleus. The pathway remained even after a lesion involving all the ipsi lateral vestibular nuclei. Hence it is concluded that the pathway with monosynaptic connexion to flexor motoneurones originates from structures outside the ipsilateral vestibular complex. That this pathway is of supraspinal origin was postulated from the results of an experiment in which an almost complete chronic ipsilateral hemisection was performed at C3—C4 level, sparing only the medial part of the ventral funicle. In the following acute experiment stimulation of the ipsilateral cord at the lower thoracic level did not evoke any monosynaptic EPSP in flexor motoneurones. In recent experiments (Grillner and Lund 1966) the supraspinal origin of the pathway with monosynaptic connexion to flexor motoneurones have been further investigated.

It is of interest also to mention that the effects in flexor motoneurones on stimulation of Deiters nucleus were mainly inhibitory with a segmental latency for the IPSP of 1.1—1.5 msec, thus indicating a disynaptic linkage.

The problem of the distribution of the vestibulospinal synapses within the somatodendritic complex of the extensor α-motoneurones is of particular interest (Fadiga and Brookhart 1960). This problem needs further experimental data but a few observations are available from the present material. The values for the "time to peak" and the decay of the monosynaptic EPSP induced in extensor motoneurones by single shock stimulation of the Deiters nucleus are well in accordance with the corresponding values for the homonymous group Ia EPSP. This suggests that in the cat there is no difference in the average distance from the soma of the postsynaptic membrane for the two kinds of synapses as has been found in the frog (Fadiga and Brookhart 1960).

There is some morphological evidence that fibres of the vestibulospinal pathway in the cat terminate on the ventral horn cells (Schimert 1938, Carpenter 1960, Staal 1961) but no distinction was made between motoneurones and other types of cell located in the ventral horn. Recent anatomical studies (Nyberg-Hansen and Mascitti 1964) indicate that the vestibulospinal tract terminates exclusively on cells in the entire lamina VIII and the neighbouring regions of lamina VII of Rexed (1954) but not in layer IX where the motoneurones are situated. Scattered degenerated fibres have, however, recently been observed with silver impregnation technique (Erulkar *et al.* 1966) but it may be that only observation of degenerating fibres with electrone microscopy can definitely contribute to the solving of this problem. It should be conceded from the findings of Nyberg-Hansen and Mascitti (1964) that at least some of the vestibulospinal fibres terminating in laminae VIII and VII form axodendritic contacts with lumbar motoneurones. These dendrites may actually extend for a considerable distance from their perikaryon and even beyond the confines of the lamina in which it is situated (Sprague and Ha 1964). On the other hand the interneurones responsible for the disynaptic EPSP on the extensor and IPSP on the flexor motoneurones are likely to be located in the above mentioned laminae VIII and VII.

The [illegible] the [illegible] pathway [illegible] link in the mechanism controlling the extensor motoneurones is supported by several studies (see Brodal *et al.*, 1962). Following stimulation of the lateral vestibular nucleus in the cat Sasaki *et al.* (1962) recorded two separate groups of EPSPs from extensor motoneurones of the hindlimb, while the effects on the flexor motoneurones were insignificant. The early group was set up at a latency of about 1.0 msec, lasting for a relatively short time and was present in all motoneurones of [illegible] The late group, on the other hand, showed a latency period of 1.5 to 2.0 msec or more and had a long duration with gradual rise and fall. Even the gradual depolarization due to this late group was seldom capable of firing the motoneurones. As pointed out in our study the monosynaptic EPSP induced from Deiters' nucleus has a latency of about 3.0 msec [illegible] (Sasaki, 1962) and is subthreshold for firing.

With respect to [illegible] peripheral inputs it is of interest that both labyrinthine [illegible] [illegible] Pompeiano and Cotti [illegible] and spinal afferents (Giaquinto *et al.*, 19[illegible]) [illegible] neurones of Deiters' nucleus. It is also of interest [illegible] [illegible] group Ia afferents do not affect the activity of the Deiters' neurones (Giaquinto *et al.*, 1963). This finding, which has been obtained in [illegible] [illegible] with the cerebellum intact, has recently been confirmed by Wilson *et al.* (196[illegible]). On the other hand, the cutaneous and the high threshold muscle afferents, which are responsible for the flexion reflex (Eccles and Lundberg, [illegible]) exert powerful excitation on Deiters' neurones (Giaquinto *et al.*, 19[illegible]; Wilson *et al.*, 196[illegible]).

[illegible] upon Deiters' nucleus, it should be recalled that both the uncrossed and crossed fastigiobulbar fibres are able of exerting respectively [illegible] rostrolateral Deiters' nucleus (cf. Brodal *et al.*, 1962). The [illegible] degree [illegible] localization in these pathways (Walberg *et al.*, 19[illegible]) [illegible] as [illegible] spinal projection from Deiters' nucleus (Pompeiano and Brodal, 1[illegible]) [illegible] the excitatory effect from the fastigial nucleus [illegible] direct [illegible] vestibulospinal pathway may be highly localized.

Of particular relevance is the fact that the vestibulospinal pathway is involved in the [illegible] of the cerebellar inhibition of extensor motoneurones. It has been [illegible] shown [illegible] the rostrolateral part of the fastigial nucleus abolishes the [illegible] the decerebrate rigidity elicited by repetitive stimulation of [illegible] vermis of the anterior lobe (Moruzzi and Pompeiano, 195[illegible]). Following [illegible] demonstration that the long corticofugal fibres to Deiters' nucleus [illegible] vermal [illegible] of the anterior lobe pass through the rostrolateral part [illegible] nucleus (Walberg and Jansen, 1961; Eager, 1963), the suggestion has [illegible] forward that these long cerebellar cortico-vestibular fibres are responsible [illegible] inhibition of Deiters' neurones (Brodal *et al.*, 1962, pp. 15[illegible]). [illegible] experiments with intracellular recordings have clearly shown that stimulation [illegible] cerebellar vermis of the anterior lobe gives monosynaptic IPSP on the Deiters' [illegible] and Kawai, 1964; Ito and Yoshida, 1966; Ito *et al.*, 1966).

The collapse of the extensor rigidity induced by high frequency stimulation of the vermis of the anterior lobe is therefore due to inhibition of tonogenic centres (particularly the Deiters nucleus) which are responsible for decerebrate rigidity. In agreement with this hypothesis is the fact that the hyperpolarization of the extensor motoneurones induced by stimulation of the vermis of the anterior lobe is not reversed by passing hyperpolarizing current through the membrane, nor is it associated with the conductance changes which determine the IPSP (Terzulo 1959, Llinás 1964). The conclusion from these observations was that the cerebellar inhibition is due to suppression of tonic excitatory influences acting upon the motoneurones.

The present demonstration that Deiters' nucleus exerts a monosynaptic excitatory influence on the extensor motoneurones leads to the conclusion that the inhibition of the decerebrate rigidity induced by cerebellar stimulation is due to a striking disfacilitation of the extensor motoneurones as a consequence of an abrupt suppression of the vestibulospinal excitatory input to the extensor motoneurones. It is of interest that even the projection pathway from the cerebellar cortex to Deiters' nucleus is highly localized (Pompeiano and Cotti 1959, Walberg and Jansen 1961, Voogd 1964).

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Added in proof

1. A later investigation of these descending monosynaptic effects (Grillner, Hongo and Lund, to be published) a greater variability was found in that in some animals the average descending EPSP was considerably smaller than in others and hence, from a quantitative point of view the present results may not be representative for all animals. However no animals has been found in which the descending effect is entirely lacking, and conclusions drawn from the lesion experiments presented above are not endangered.

2. In recent experiments in our laboratory it was observed that stimulation in the lower thoracic region of the VLF evoked a monosynaptic EPSP in a few motoneurones (ten Bruggencate, Burke and Lundberg, personal communication). Hence the findings by Willis et al. (1967) are confirmed. In the present experiments the effects from the VLF were investigated only in cats with supraspinal chronic lesions, but it is not known if this is the explanation for our failure in observing this action, or whether the [illegible] effect is caused by a rare synaptic connection present only in some animals.

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Exclusive Thalamic Location of Subcortical Spontaneous Barbiturate Spindles

By

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Abstract

JUNGE, K. and O SVEEN. *Exclusiv thalami locat n of subcortical spontaneou ba bitu at pindl* Acta physiol sc nd 1968 73 22—31

Spontaneous discharges of neurones in the thalamu the mesencephalic nd pontine ret ular formation, the caudate nucleus and the hypothalamus were ecorded in ats anesthetized th barbit rates Onl thalamic lls showed the pindle pa tern of discharges, onsisting of high frequencies bursts sepa ted by silent intervals, orresponding to repetition frequency of 7—14/sec All neurones outside the thalamus discharged randoml w th no correlation to the simultaneousl occurring thalamic spindle activit It is concluded that subcortical neurones participating spontaneous barbitur s spindle activit ha an exclusiv thalamic lo tion Consequentl the processes of synchronisa ion nd des nchronisa ion both most likely take place at thalamic lev l

There is good evidence that cortical barbiturate pindles (Derbyshire *et al* 1936, Bremer 1937) are due t pacemaker located in the thalamus (Bremer 1935 1937 1958) Th cortical pindles re not abolished by a complet section of the brain stem at a pre-coll ular level, thu indicating their independ nce of mesencephalic connections Later Adri n 1941) rec rded pontaneous potentials from thalamocortical axons lose to their termination in the cortex The pot ntials consisted of groups of waves with the same rh thmic characteristics as the cortical spindles. These thalami sp ndles persisted after decortication, uggesting that the 8—12 sec cortical rhythmic t t ould be triggered by thalamic activity From a series of experiments Dempsey nd M rison oncluded that the intralam nar and midline nuclei of the thalamus were essential in the production of 7—14 sec spindle activity in the cortex (Morison nd Dempsey 1942 Dempsey and Morison 1942a, b) This view was strengthened when M rison, Finley and Lothrop (1943) found spontaneous slow wave rhythmical t t to be most prominent thin the unspecific nuclei of the thalamus, especiall near to or within the centre median. When the unspecific thalamic nuclei were st m lated, the resulting cortical recruiting potentials mimicked the spontaneous spindle a t ty (Dempsey and Morison 1942a Jasper 1949) The

work of Jasper (1954) confirmed the view that the midline and intralaminar thalamic nuclei were essential in the production of 8—12 sec cortical activity. These nervous structures are closely related to the mesencephalic reticular formation (Jasper 1954). More recently it has been shown (Andersen and Sears 1964, Andersen, Andersson and Lømo 1967a, b) that most nuclei in the thalamus, including the specific relay nuclei have the ability to produce 8—12/sec spindle activity by themselves. Removal of the unspecific thalamic nuclei did not seem to have obvious effects upon the rhythmical activity in the specific thalamic nuclei. It could be argued that the rhythmic activity is initiated further caudally and transmitted to the thalamic level by ascending impulses. The aim of the present investigation has been to study the possible relationship between the activity of the thalamic nuclei and cells of the mesencephalic reticular formation, by a determination of the distribution of subcortical spindle activity. Single units in the thalamus, the hypothalamus, the caudate nucleus and the mesencephalic, pontine and bulbar reticular formation were classified according to their pattern of firing with particular reference to activity at about 10 sec. In addition, it was observed whether neuronal activity outside the thalamus (spindle pattern or random firing) exhibited any constant behavior just preceding the thalamic spindle to suggest a role as spindle initiator or pacemaker.

Methods

Adult cats were used, lightly anesthetized with sodium pentobarbital (Nembutal, 30 mg/kg, i.p.). Additional doses were administered i.m. in doses not exceeding 10 mg to maintain the anesthesia level where the cat first withdrew the forepaw when pinched.

The neocortex and the hippocampal formation were removed by suction, exposing the dorsal surface of the caudate nucleus, the thalamus and the superior colliculus on the left side. For recording glass micropipettes were used (4M NaCl, 1—2 MΩ) usually 4 at a time. Two of them were inserted into the thalamus, and two into the reticular formation or the caudate nucleus, each pair carried by a micromanipulator. The signals were fed into a quadruple cathode follower and conventional AC-amplifiers, displayed on a cathode ray oscilloscope and photographed. Sometimes a dual channel ink writer was used for display. The insertion of the microelectrodes was guided by direct vision of the structures involved, with additional aid from Horsley-Clarke coordinates. In some experiments, the marking technique described by Thomas and Wilson (1965) was used, with subsequent histological control.

Results

Firing pattern of mesencephalic reticular cells. In these experiments, two microelectrodes were first inserted into the thalamus, recording spontaneous barbiturate spindles. An extracellularly recorded spindle may be described as a series of grouped, high frequency cell discharges occurring 7—14 times a second. The burst discharges are interrupted by positive waves. Sometimes, the grouped cell discharge expected between two successive positive waves, may be absent. A spindle, consisting of alternating negative and positive waves, usually lasted for 1—3 sec. While such spindles recurred regularly in the thalamus, the mesencephalic reticular formation was explored, usually with two microelectrodes at a time.

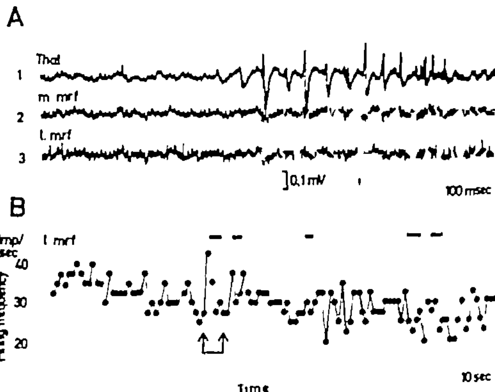


Fig. 1. A. 1 pe l ac n ty of thalamus Thal ll and mesencephalic re lar formation (mrf) ecorded simult neousl during fu bit rat anesthesia. B. Discha ge frequency of same ell show 4 as 3 I rval ma ked by rrows corresponds I II as lines fi t oc ence of halamic spindles

In th upper t ac of F g 1 4 is characteristic spindle that wa rec rded from th e entrobasal complex f th thalamu The two lower tracks re simultaneous record from the medial and lateral part of th mesencephalic ret lar formation (m. mrf and l mrf) respectivel In the lateral retic lar trace, a ingl cell discharged randomly without ny const nt change in the f ring pattern bef re d ring or after th thalamic spindl In Fig 1 B the discharge frequency of th same reticular formation ll i plotted gainst time The number of discharges were counted in peri d of 400 msec and expressed as impulses per second The nterval between the arrow is the period show In Fig 1 4 The horizontal lines ndicate the occurrence of pindles as recorded by one of th thalamic electrodes Th reticular cell showed a considerable variation f it discharge frequency However this variation was not correlated with the occurrence of spindles in the ventrobasal complex of the thalamu Concluding from 12 similar experiments with a total of 50 microelectrode tracks through the thalamus no correlation was found between the firing pattern of any of the mesencephalic neurones and th spindle activ ty of any thalamic location.

In Fig. 2, the two records were taken simultaneously from the thalamus and the

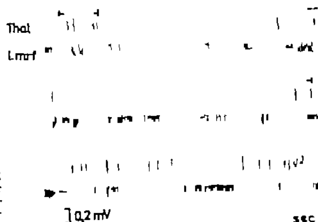


Fig Activity of thalamic cell and mesencephalic reticular formation cell simultaneously recorded. Thalamic spindles are indicated by the broken lines.

lateral mesencephalic reticular formation (Thal and L mrf respectively) d e to a short amplifier time constant, only spike potentials are seen. The variation of the spike amplitude of th reticular cell was due to respiratory movements. The thalamic spindles are marked with broken lines above the trace. No correlation is seen between the firing pattern of the reticular cell and the frequently appearing spindles in the thalamus

In order to demonstrate the difference in firing pattern between thalamic and mesencephalic reticular cells, histograms of the intervals between cell discharges in th thalamus (Thal) and in the lateral part of th mesencephalic reticular formation (l mrf.) were plotted (Fig 3) The interval histogram of the discharges of one thalamic cell that participated in typical spindle activity is shown in *A* and *B* Most of the intervals were shorter than 10 msec. This corresponds to the pike intervals within one burst discharge (Fig. 3 *B* filled triangles) No spike intervals between 10 and 70 msec were observed. This corresponds to the inhibitory period between the s ccessiv burst discharges (Andersen and Eccles 1962, Andersen and Sears 1964) Th group of intervals of 80—.50 msec corresponds to the silent interval between two post inhibitory rebound burst discharges of th thalamic cell (open circle, filled diamond and square) In Fig. 3 *A* the spike interval histogram of th same thalamic cell is plotted on another time scale with the corresponding histogram of reticular formation cell discharges. The ery long interspike intervals of the thalamic cell (5—15 sec) represent the silent periods between two successive spindles. The reticular cell did not show any comparably long interspike period. Its discharge frequency remained markedly constant at about 20/sec In ddition to the absence of the long silent periods, no bimodality of the spike interval distributi n was observed, contrasting the behaviou of the thalamic cell.

For a comparison, th interval histograms of the spontaneous discharges of three different reticular cells were plotted (Fig 3 *C*) including the one presented in F'g 3 *A* Although al lthree neurones had different firing frequencies, all th histograms

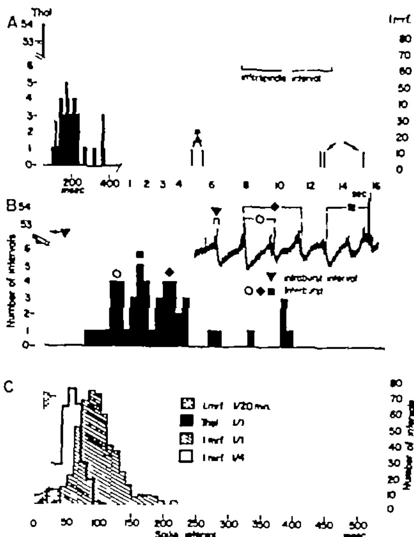


Fig. 3. A: Interval histograms of the spontaneous discharges of a thalamic cell (black) and reticular formation cell (line). B: Interval histogram of the same thalamic cell discharges as A, plotted on a faster time scale. C: Interval histograms of spontaneous discharges of 3 different reticular formation cells, including the one presented in A.

showed an unimodal distribution of the discharge interval. All the 50 cells studied in the mesencephalic reticular formation exhibited principally similar firing patterns to that of the three cells plotted in Fig. 3 C.

Fig. 4 B is a drawing of a nearly sagittal section about 5 mm from the midline of the brain stem of one of the experimental cats. In the thalamus one electrode track is shown (stippled line). The same track is magnified in Fig. 4 A. Each filled circle gives the position of a thalamic cell that discharged with the spindle pattern described above. New cells were identified by standard single unit criteria. This identification

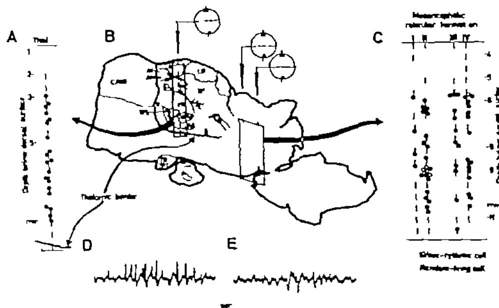


Fig. 4 *A* and *C* Distribution of spontaneously active cells recorded by microelectrodes penetrating the thalamus and mesencephalic reticular formation, respectively along the tracks indicated in the nearly sagittal section of the brain stem of one of the experimental cats in *B*. A filled circle indicates group of neurones discharging in typical spindle pattern, open circles indicate random firing *D* and *E*. show thalamic spindles recorded with the microlectrodes used in the mesencephalon.

technique gi es a too low estimate of the number of rhythmically acti cells because normall many neighbouring cells fire nearly synchronously within each burst discharge. Each of the filled circles in Fig 4 *A* therefore, represent a rhythmically active group of neurones.

In the thalamic electrode track of Fig. 4 *A* *B*, 22 different foci of rhyltmic activity were observed, using the criteria gi en above. In this particular experiment, no spontaneously active cells were found ventral to the thalamus. However this was not the rule The lack of cells showing spindle activity in the dorsal 3 mm of the thalamus that sometimes occurred (Fig. 4 *A*) may be due to local circulatory damage caused by the removal of the pia. This was necessary because th microelectrodes otherwise could not penetrate this rather tough membrane.

Sometimes some cells in the thalamus did not show spindle activity even when the majority of cells participated in the rhythm. No such cell was observed in the track ill trated n Fig 4 *A*

In Fig 4 *C* the distribution of spontaneously acti e neurones found along 4 tracks through the mesencephalic reticular formation is plotted. Th tracks were oriented n a rectangle w th one pair of electrodes 1 mm rostral to the other The distance between the electrodes in the mediolateral direction was 1.5 mm. Because of the

vertical insertion of the electrodes, the deepest parts of track III and IV were in the pontine reticular formation. Each open circle in Fig. 4 C indicates a cell that fired spontaneously but without the firing pattern described for spindle activity. In this region all cells encountered were easily isolated, using ordinary single unit criteria. No tendency to stimulate discharge of neighbouring neurones was observed. As noted earlier this firing pattern was the only one observed for reticular cells. As may be seen from the cell density in Fig. 4 C there was no difficulty in finding cells with such a firing pattern.

The records in Fig. 4 D and F were obtained from the thalamus with the same two microelectrodes that were used to obtain the data of Fig. 4 C indicating that the lack of spindle activity in the mesencephalon was not due to adverse recording conditions.

Firing pattern of caudate cells. Since there was no correlation between single cell activity in the mesencephalic reticular formation and the thalamus a possible relation between the firing pattern of caudate and thalamic cells was looked for. The main reasons for studying the caudate nucleus were that the caudate nucleus has well developed connections in both directions with thalamic nuclei, and that cortical spindles are reported to be started by single shocks delivered to the caudate nucleus (Buchwald *et al.* 1961). These facts could suggest rhythmic spindle activity of caudate neurones.

The upper trace in each pair of Fig. 5 A and B was recorded from the thalamus. Cell activity with burst discharges and slow waves was observed. The lower trace recorded from different locations within the head of the caudate nucleus. In the 5 cats used for this purpose 20 cells were studied, all of them having a firing pattern similar to that shown here. After finishing the caudate recording the microelectrode was moved to the thalamus where spindle activity easily was recorded (Fig. 5 C).

In Fig. 5 D are histograms showing the intervals of the spontaneous discharges of a caudate neurone (dots) and of a thalamic cell (hatching). The thalamic histogram is the same as in Fig. 3 and the caudate histogram is from the cell shown in Fig. 5 B. The fundamental difference between the two histograms is the unimodal distribution of the caudate spike intervals, contrasting the bimodal distribution of the thalamic discharge intervals. These findings are in accordance with observations of Rocha Miranda (1965) and suggest that the caudate nucleus can hardly be regarded as a pacemaker for the 10/sec activity found in the thalamus.

It was frequently observed that the thalamic spindles disappeared at a depth of about 9 mm below the dorsal surface of the thalamus, corresponding to the border to the hypothalamus. Sometimes randomly occurring cell discharges were found ventral to this border but the spindle discharge pattern was never seen. In one instance the depth at which spindle activity disappeared was marked with fast green (Thomas and Wilson 1965) and found to correspond precisely to the border between the thalamus and the hypothalamus.

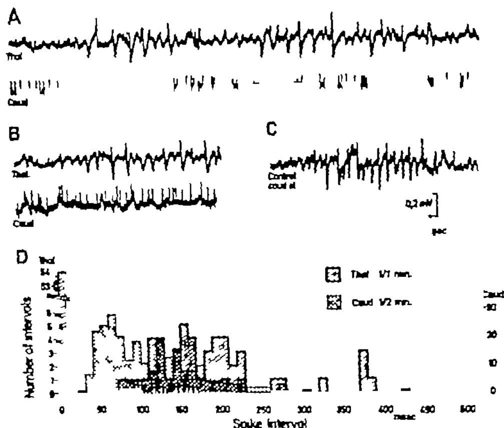


Fig. 5. *A* and *B*. Recording of different spontaneously active thalamic (Thal.) and caudate (Caud.) cells. *C*. Control record of thalamic spindle with the microelectrode previously used in the caudate nucleus. *D*. Interval histogram of spontaneous discharges of thalamic (hatched) and caudate (dotted) neurone.

Discussion

Spontaneous barbiturate spindle activity with rhythmically grouped cell discharges about 10/sec. was only found in the thalamus. Since none of the more than 250 reticular formation cells investigated showed any spindle discharges in the range of 7—14/sec. the result is probably representative. The lack of spindles recorded from extrathalamic sites was not dependent upon a failing of the recording system or the condition of the animal. Since no spindle activity occurred in the mesencephalic reticular formation, in the caudate nucleus, or in the hypothalamus, under conditions when such activity was prominent in the thalamus, the main conclusion is that *the rhythmic spindle activity is an exclusive thalamic property.*

Of particular interest was the abrupt cessation of rhythmically active cells as soon as the electrode passed from the thalamus into the hypothalamus. Since the rhythmic activity occurred with regular intervals down to the point of disappearance, the

returned on withdrawal of the electrode the difference in firing patterns wa probably real.

The fact that *no spindle activity was found in the caudate nucleus* is interesting when compared to the observations of Buchwald *et al.* (1965) who found that cortical spindles can be triggered by single shock stimulation to the caudate nucleus. The most reasonable explanation seems to be that a synchronous *afferent volley to the thalamus may start a thalamic spindle in a similar way to that described by Andersen and Sears 1964 with the use of peripheral or antidromic stimulation. Ac*tivity in the caudate nucleus can probably only modif not in tiate spindle activity

Jasper and his collaborators (Ja per 1954 1958 Jasper and Ajmone-Marsan 1952) maintained that the intralaminar and medially located nuclei of the thalamus can be regarded as the rostral extension of the reticular acti ating system. It was further postulated that *ascending acti ity from the reticular formation activates* medial areas of the thalamus From here the activ ty will proceed to other thalamic nuclei and then to the cortex Because rhythmic *7—14 sec* activity is found only within the confines of the thalamus and cortex, the ascending infl ence from lower parts of the brain stem mu t be of a different nature and can thus only mod f the rhythmic activity that is created w thin the thalamus itself Since des nchronization of the lectroencephalogram by def nition is a disruption or blocking of s nchronous *7—14 sec* activ ty it follows *that the mechanisms behind synchronization and desynchronization* should be studied w thin the thalamus proper Lower part of the in stem w th it pecific as well as non-specific afferent systems must therefore be rded as capable of modif ing the thalamic rhythmic acti ity only and not as the erator of the rh thmic acti it nor as the sit of abolition of rh thmicity during t nchronizat on

W wish hank Dr Pe Andersen for his luable help and riticism and D Eric Rin k for aluable ana omic l help

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Secretory Responses and Respiratory Enzymes of the Rat's Submaxillary Gland after Repeated Teeth Amputations

By

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Abstract

Ohlin P and C J Perec. *Secr tory responses and respiratory enzymes of the rat s ubma illary gland afte repeated teeth amputation* Acta physiol scand. 1968. 73 3 35

The ffec of pe ted teeth mput tions on the secretory responses and the activi ies of suc nic del drogenase o hrome oxidase and fumarase was studied in the rat s subm xillary gland. Repe ted eeth mputations ave an enlargemen of the land. The secretory responses to ad enaline w re found he increased while hose to acetylcholine methacholine or stimu tion of l pa mpa he ic secretory ners were unchanged The total activ ties of succ nic h drog nase nd f ma se were ncre sed while tha of ytochrome oxidase was unchanged

A gain w gl t of the rat ubmaxillary gland follows repeated teeth amputations (Wells t al 19?9) It wa thou ght t be d e to a reflex stimulation of the gland via the t x ic nerves nduced by the constant irritation of receptors within the pulpa nd dj nt n u sa after teeth a nputations The glandular enlargement corresponds hist log all t an increase in size f the acinar cells while the duct cells seen t be unaffected (Perec t al 1965) Very little is known about the function of the gland aft r repeated teeth amputations Therefore t seemed of interest to t id the f ncti n f nlarged glands, particularly since it is known to be changed after other t t al xod fic ti ns (see Ohlin 1966) For thi purpose the secretory responses t lagogue agent and nerve timulation were studied A another indicator of the f ct n the act ties of some respiratory enzymes, succinic dehydrogenase cytoch o e xid se and fumarase were determined

Methods

64 female rats, 4—5 months old and wei h ng abou 200 g were used. They w re given pelleted diet (Anticimex 210 nd w *ad l bit m* The ts w h amputated teeth received crushed pellet in milk.

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22 rats w re used for the study of secretory responses 9 out of these animals were untreated controls. Teeth amputations were made by cutting the inferior incisors with small bone-forceps daily for week. — The rats were anesthetized with chloralose (100 mg/kg) i.v. after preliminary ether The submaxillary ducts were exposed in the neck and cannulated using small glass cannulae which gav about 80 drops out of 1 ml of distilled water Secretion ppearing t the tip of the cannula was marked on smoked drum. T estimate the threshold doses and the secretory responses to sialagogue agents series of doses of acetylcholine (0 — 5 μg/kg) methacholine (0.1—10 μg/kg) adrenaline (0.5—20 μg/kg) and isoprenaline (1—100 μg/kg) were given intravenously The maximal secretory response to nerv stimulation was determined by stimulating the chorda-lingual nerv w th 20 shocks/sec, rectangular pulses with duration of 1.5 msec and of supramaximal oltage were used. It is expressed as μl saliv per min per gland or as μl saliva per min per mg glandular tissue (dry w ight)

For the determinations of the activities of respiratory enzymes 25 rats were used; 14 animals were untreated controls. The rats were killed by cervical dislocation. The submaxillary glands were carefully leaned, weighed and homogenized in redistilled w er The activities of succinic dehydrogenase and cytochrome oxidase were manometrically estimated by Warburg technique (see Ohlin 1962) and that of fumarase was spectrophotometrically determined as devised by Racker (1950) The enzyme activities were calculated in units per gland (total activity) and per g of glandular tissue (concentration) One unit corresponds to utilization of 1 μl oxygen t 37 C in 30 min for succinic dehydrogenase and cytochrome oxidase and to change in optical density of 0.001/min t 240 mμ for fumarase.

The effect of repeated teeth amputations on the weight of the submaxillary gland was studied in 17 rats 11 animals were controls. After ha ing killed the animals the wet weight of the submaxillary gland was determined after extirpation and careful cleaning. The dry weight of the gland was estimated after heating to 105—110 C for 48 hrs.

Results

Gland W ght Repeated teeth amputations for a week caused an increase ($P<0.001$) of the wet weight of the rat s submaxillary gland of about 30 per cent, from 152±5.5 (11) to 204±11 (6) mg. The dry weight was also increased by about 30 per cent.

S cr tory Responses In control animals th threshold doses of acetylcholine and methacholine were found to be 0.5—? μg/kg for acetylcholine and 0.—1 μg/kg for methacholine. Doses above threshold caused a slow flow of saliva e.g after 10 μg methacholine/kg the secretory response was found to be 1— drops of saliva. The threshold doses and the secretory responses to acetylcholine and methacholine were not changed after repeated teeth amputations.

The threshold doses of sympathomimetics were found to be 1—2 μg/kg for drenaline and about 1 μg/kg for isoprenaline in controls. The secretory response to 20 μg adrenaline/kg was 1—2 drops of saliva and to 10 μg isoprenaline/kg about 1/2 drop. After repeated teeth amputations the threshold doses to adrenaline and isoprenaline were unchanged while the secretory responses to doses of adrenaline above threshold seemed increased. Thus 20 μg adrenaline/kg was found to cause a flow of 2—3 drops of saliva in animals w th amputated teeth. The secretory responses to different doses of isoprenaline were not similarl ncreased.

Stimulation of the chorda-lingual nerve w th 20 shocks/sec has previously been shown to cause a secretion of maximal rate from the rat s submaxillary gland (Ohlin 1965) The maximal secretory response to chorda stimulation was found to be

Mea ± standard error of mean (number of observations)

TABLE I Activities of Respiratory Enzymes in the Submaxillary Gland of Rats after Teeth Amputations and in Controls. Values are Mean ± S.E.M.

	Number of gland	Succinic dehydrogenase activity		Cytochrome oxidase activity		Fumarase activity	
		per gland	per g gland tissue	per gland	per g gland tissue	per gland	per g gland tissue
Controls	16	845 ± 42	5370 ± 253	3210 ± 143	20,400 ± 1190	2450 ± 135	15 650 ± 952
Teeth amputations	22	1130 ± 40*	6100 ± 241	3170 ± 150	17 190 ± 969*	2840 ± 181	15,500 ± 835

P<0.001 *P* 0.05 when compared with *Controls*

46±2.4 (9) μl per min per gland or 1.11±0.07 (9) μl per min per mg glandular tissue (dry weight) in the controls. After repeated teeth amputations the maximal secretory response was not significantly changed when expressed per gland: it was 40±1.6 (7) μl per min per gland. When calculated per unit weight, it was decreased ($P<0.001$), however, to 0.82±0.04 (7) μl per min per mg glandular tissue.

Respiratory Enzymes. The total activity of *succinic dehydrogenase* was found to be significantly increased by about 40 per cent after repeated teeth amputations. The enzyme concentration was also increased, though only by 15 per cent (Table I).

After repeated teeth amputations the total activity of *cytochrome oxidase* was unchanged. The enzyme concentration was significantly decreased by about 17 per cent (Table I).

In the enlarged gland the total activity of *fumarase* was found to be increased in relation to the gain in weight obtained after repeated teeth amputations. The enzyme concentration was therefore unchanged (Table I).

Discussion

Repeated teeth amputations did not affect the secretory responses of the *rat* submaxillary gland to parasympathomimetics or to stimulation of the parasympathetic secretory *nerve* in spite of the marked gain in weight of the gland. Since the glandular enlargement is due to an increase of the acinar cells (Perec *et al.* 1965) the present results suggest that the acini do not play an important role for the secretory responses to parasympathomimetics, which agrees with previous findings indicating that the tubules seem to regulate the parasympathetic secretion (see Ohlin 1966).

The submaxillary gland of rats is supplied with secretory receptors for catecholamines of both α- and β-type, the α type is dominating (Ohlin 1964, Emmelin *et al.* 1965). It was found that the secretory responses to adrenaline were increased in

relation to the gain in the size of the glands after repeated teeth amputations which indicates that the acini may be of importance for the sympathetic secretion. It is of interest to note that the acini but not the tubules of the rat's submaxillary gland have an adrenergic innervation (Norberg and Olson 1965).

The main metabolic pathway in the rat's submaxillary gland seems to be via the tricarboxylic acid cycle (Goldman *et al.* 1964). Two enzymes of this cycle were studied and the enzyme activities were found to be increased after repeated amputations. On the other hand, the total activity of another respiratory enzyme, cytochrome oxidase, was unchanged.

The enlargement of the rat's submaxillary gland after repeated teeth amputations is dependent on the sympathetic and parasympathetic glandular nerves (Wells and Peronace 1964, Ohlin and Perec 1967). It seems likely that not only the gain in gland weight after repeated teeth amputations but also the increased activities of succinic dehydrogenase and fumarase are dependent on the glandular nerves, very likely the parasympathetic nerve since parasympathetic denervation of the rat's submaxillary gland decreases the activities of these enzymes (Ohlin 1963 b). The increased enzyme activities may also play a part in the development of the glandular enlargement after repeated teeth amputations since a close relationship has previously been shown between the activities of respiratory enzymes and the size of salivary glands in cats and rabbits (Nordenfelt *et al.* 1960).

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TABLE I. Activities of Respiratory Enzymes in the Submaxillary Gland of Rats after Teeth Amputations and in Controls. Values are Mean ± S.E.M.

	Number of glands	Succinic dehydrogenase activity		Cytochrome oxidase activity		Fumarase activity	
		per gland	per g gland tissue	per gland	per g gland tissue	per gland	per g gland tissue
Controls	16	845 ± 42	5370 ± 253	3210 ± 143	20 400 ± 1190	2450 ± 133	15,650 ± 932
Teeth amputations	22	1150 ± 46*	6100 ± 241*	3170 ± 150	17 190 ± 989*	2840 ± 181*	15 500 ± 835

* P<0.001 *P<0.05 when compared with Controls

46±2.4 (9) μl per min per gland or 1.11±0.07 (9) μl per min per mg glandular tissue (dry weight) in the controls. After repeated teeth amputations the maximal secretory response was not significantly changed, when expressed per gland: it was 40±1.6 (7) μl per min per gland. When calculated per *unit weight*, it was decreased (P<0.001), however, to 0.82±0.04 (7) μl per min per mg glandular tissue.

Respiratory Enzymes. The total activity of *succinic dehydrogenase* was found to be significantly increased by about 40 per cent after repeated teeth amputations. The enzyme concentration was also increased, though only by 15 per cent (Table I).

After repeated teeth amputations the total activity of *cytochrome oxidase* was unchanged. The enzyme concentration was significantly decreased by about 17 per cent (Table I).

In the enlarged gland the total activity of *fumarase* was found to be increased in relation to the gain in weight obtained after repeated teeth amputations. The enzyme concentration was, therefore, unchanged (Table I).

Discussion

Repeated teeth amputations did not affect the secretory responses of the rat's submaxillary gland to parasympathomimetics or to stimulation of the parasympathetic secretory nerve in spite of the marked gain in weight of the gland. Since the glandular enlargement is due to an increase of the acinar cells (Perec *et al.* 1965), the present results suggest that the acini do not play an important role for the secretory responses to parasympathomimetics, which agrees with previous findings indicating that the tubules seem to regulate the parasympathetic secretion (see Ohlin 1966).

The submaxillary gland of rats is supplied with secretory receptors for catecholamines of both α- and β-type, the α-type is dominating (Ohlin 1964, Emmelin *et al.* 1965). It was found that the secretory responses to adrenaline were increased in

while in the latter reduction of the tidal volume will increase uneven distribution of ventilation and even lead to closure of alveoli and increased venous admixture

The purpose of this article is to report on the relationship between A—aDO$_2$ and the alveolar ventilation, represented by PaCO$_2$, in anesthetized dogs breathing air the changes of the alveolar ventilation being achieved by varying the tidal volume keeping the respiratory rate constant.

Materials and methods

Nine studies were performed on nine healthy mongrel dogs, weighing 10—23 kg, with an average hemoglobin concentration of 12.4 g/100 ml. General anesthesia was obtained by the intravenous administration of 26 mg/kg body weight of sodium pentobarbital additional doses being administered repeatedly whenever spontaneous respiratory activity and fighting of the respirator occurred. The dogs were tracheotomized and ventilated with room air by means of volume cycled respirator The alveolar ventilation was changed every 20 minutes by varying the tidal volume keeping the respiratory rate constant, at 16—22 per minute

Expired air was collected over a 3 minute period in Douglas bag at the end of each 20 minute period of steady ventilation. During the last minute 5 ml blood was collected in heparinized Luer syringe.

The CO_2 and O_2 concentrations of the expired air were determined with the 0.5 ml Scholander Gas Analyzer (Scholander 1947) The blood PO_2 was determined polarographically at 37.5 C with Beckman Physiological Gas Analyzer using a Beckman Macro Electrode in Freeman Bradley constant temperature water bath. The blood PCO_2 was calculated by means of the Henderson-Hasselbalch equation, inserting the pH measured with the Radiometer Micro Electrode and the CO_2 content of anaerobically separated plasma, measured in manometric Van Slyke Apparatus PAO was calculated by means of the alveolar air equation, inserting the arterial for the alveolar PCO and A—aDO$_2$ was calculated as the difference between PAO$_2$ and PaO$_2$ (Riley *et al* 1946)

The venous admixture was calculated in accordance with the standard shunt equation (Riley and Cournand 1951) assuming that the A—aDO$_2$ was solely due to venous admixture, that the O capacity of the hemoglobin was 17 ml/100 ml and the solubility coefficient of O_2 in plasma was 0 003 ml/mm Hg and that the average arterio-venous difference was 20 or 40 in terms of hemoglobin O saturation applying the recently proposed standard hemoglobin dissociation curves of Severinghaus (1966)

Results

The arterial blood gases and the expired air gases were determined at 3—5 levels of ventilation in 9 dogs. PaCO varied between 22 and 80 mm Hg and PaO between 109 and 21 mm Hg.—Fig 1 shows the relationship between A—aDO$_2$ and PaCO$_2$ as observed in the 9 expts. A—aDO$_2$ decreases with increasing PaCO$_2$ in all experiments At PaCO$_2$ below 30 mm Hg all A—aDO$_2$ observations are higher than 15 mm Hg, while at PaCO$_2$ above 50 mm Hg all values are lower than 15 mm Hg The observations were divided in 3 groups according to PaCO$_2$ group 1 30 mm Hg, group 2 31—50 mm Hg and group 3 51 mm Hg and the average relationships between PaCO$_2$ PaO$_2$, PAO$_2$ and A—aDO were calculated for each group.—The averages appear from Fig 2 In group 1 (mean PaCO$_2$ 25 5 mm Hg A—aDO is 28 3 mm Hg (range 18—42 mm Hg In group 2 (mean PaCO 41 mm Hg) A aDO is 19 3 mm Hg range 13—30 mm Hg) and in group 3 mean PaCO 60 5 mm Hg) A aDO is 9 7 mm Hg range 5—14 mm Hg —The average PaO for the three groups are 91.2, 77 2 and 55 7 mm Hg

at low PAO_2 could when not taken into consideration, lead to an overestimation of the venous admixture. However the diffusion dependent component of A—aDO at low PAO_2 is probably much smaller than previously assumed (Asmussen and Nielsen 1960, Staub 1961, Staub *et al.* 1961) and the deletion of this factor in the calculations has probably not influenced the estimates of the venous admixture to a significant degree.

The calculation of the venous admixture is based on the assumption that the function of the lungs can be divided into two compartments, one with ideal gas exchange and one with a ventilation/perfusion ratio of zero. The estimate of the extent of lung tissue with impaired alveolar arterial gas exchange thus obtained is less than if a physiologically more correct model with less extreme disturbances of the ventilation/perfusion ratios were applied (Riley and Permutt 1965).

The data are different from those which would be found when the changes in PAO are obtained by varying the O concentration in the inspired air (Lilienthal *et al.* 1946, Rahn and Farhi 1964, Richards 1965). A decrease in PAO due to inhalation of low O gas mixtures is accompanied by hyperventilation and a more even distribution of ventilation, and a decrease in venous admixture, leading to an even more marked fall in A—aDO with decreasing PAO than in the present series. The alveolar hyperventilation leads to respiratory alkalosis and is thus probably associated with other patterns of distribution of ventilation and perfusion than in this series, with respiratory acidosis. Addition of CO to the low O gas mixtures would avoid the alkalosis but lead to an even more vigorous hyperventilation and evening of the distribution of ventilation.

The presented data, together with the above considerations show clearly that the absolute figures of A—aDO should only be used for the evaluation of changes in the alveolar-arterial gas exchange when the alveolar ventilation, as well as PaCO and PAO_2, remains unchanged. Serious misinterpretations may ensue if the dependency of A—aDO on these factors is not taken into consideration.

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The Relation between the Action Potential and the Active State in Human Fetal Myocardium and its Dependence on Muscle Length and Contraction Frequency

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Abstract

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Active state and action potential measurements were performed on papillary muscles from human foetuses. Changes in these two parameters induced by alterations in muscle length and contraction frequency were studied. Shortening of the muscle not only reduced the intensity of the active state, but also shortened its time course without any concomitant alteration in action potential duration. A decrease in stimulation frequency caused a reduction in the intensity of the active state and an increase in the durations of the active state and the action potential. The change in the action potential duration was considerably greater than that of the active state. The results are discussed in the relation to the activator hypothesis for excitation-contraction coupling in the myocardium.

During the last few years much new information has become available concerning the excitation-contraction coupling in heart muscle. (For reviews, see Fleckenstein 1964, Brady 1964, Edman 1965.) Papillary muscle preparations from cat, rabbit and guineapig have proved particularly suitable for both mechanical and electrophysiological studies. The purpose of the present investigation was to extend these studies to a human myocardial preparation, the human foetal papillary muscle. Early in this investigation it was found that the length (Frank 1895, Starling 1918) and frequency (Bowditch 1871) dependence of myocardial contractile strength also applied to this preparation. Experiments were therefore undertaken to elucidate the mechanisms of these inotropic interventions. It was considered desirable to study the active state instead of the more conventional recording of the isometric twitch tension. The intensity of the active state at a given moment was measured as the shortening capacity of the contractile unit at that very instant.

The form of the force-velocity curve, obtained at a given length of the contractile unit in rabbit papillary muscle, is virtually unchanged throughout a contraction cycle (Edman and Nilsson 1968) This means that a given increase in the maximal shortening velocity of the preparation is accompanied by an equivalent increase in its maximal capacity to produce tension. Thus, the shortening velocity may be used as an index of the intensity of the active state, as originally defined by Hill (1938)

It was recently shown in papillary muscles from rabbit that changes in muscle length not only alter the maximal intensity of the active state, but also markedly influence its time course (Edman and Nilsson 1968) As will be demonstrated in the present investigation, the change in the time course of the active state so produced is not attributable to a change in the duration of the action potential.

The positive inotropic effect of an increase in stimulation frequency in the myocardial ventricle is known for frog and most mammalian species. Abundant evidence supports the view that changes in active state intensity produced by this inotropic intervention are not related to any alteration of the action potential amplitude. (For references, see Koch-Weser and Blinks 1963) Several authors have, however noted a positive correlation between the duration of the action potential and the time course of the isometric twitch tension, although the changes of these two parameters are not exactly parallel (Trautwein and Dudel 1954 Niedergerke 1956) This is in line with the general idea that the action potential, both in the skeletal muscle (Sandow Taylor and Preiser 1965 Edman and Grieve 1966, Edman, Grieve and Nilsson 1966) and the myocardium (Niedergerke 1963 a, b) not only triggers the mechanical events, but also in some way regulates their time courses. It was therefore considered of interest to compare changes in the duration of the active state and of the action potential induced by an in increase in stimulation frequency

Methods

Material

Hearts were taken from human foetuses obtained at legal abortions. The abortions were performed on socio-medical grounds according to the Swedish law. In no case was there any gross malformation of the foetus. The crown-heel length of the foetuses was 23—27 cm and the estimated gestation age 18—23 weeks. Immediately after uterine evacuation the heart was placed in an oxygenated Ringer solution (composition see below) and the right ventricle was opened. Papillary muscles were dissected from the ventricle together with a piece of the ventricular wall. As a rule only one usable preparation could be obtained from each heart. The muscle was mounted on the recording device and stimulated at a constant frequency for an equilibration period of at least 30 min before the experiment was started. The isometric tension of the preparation was very stable the decline in output being less than 10 per cent over a 4-hr period. The length of the preparations was 4.5—7.0 mm and the largest diameter 0.8—1.4 mm, as measured with an ocular micrometer (×10—20)

Mechanical recording

The technique used for mounting of the specimen and recording of tension and length during contraction was similar to that described previously (Edman and Nilsson 1968) The preparation was mounted vertically in a jacketed, thermostated bath with the lower end attached to a tension transducer RCA 5734 fitted in the bath, and the tendon end fixed to a duralumin lever via a straightened steel wire The movements of the lever were recorded by a photoelectric [illegible]. The lever had an equivalent mass of 90 mg.

The compliance of the recording device was 42 μ/g and linear up to 3 g. The preload and hence the resting length of the prepar tion, was set by loading the lever ia an lasticity on the opposit side of the f lcrum. The distance between the insertions of the muscle to the upper tendon and the entricular wall, when the muscle was mou ted and the preload applied was taken as the resting length of the muscle.

The lever was locked by a releasable cat h fitted on telephone relay. Thi atch was synchronized with the stimulus impulse and could be released at any pre-set moment during a contraction of the preparation to enable isotonic shortening after a nitial isometric phase. Suitable damping of the movements of the lever was obtained by means of dashpot containing silicone oil (iscosity 4000 Cs) situated on the same side of the f lcrum as the muscle. The natural frequency of the mechanical recording system was 00—160 cps with the preparation mounted. The muscle was stimulated by supramaximal pulses of two msec duration, the time 0.1 msec ia tw pairs of platinum lectrodes. The signals from the tension transducer and the photo-electric cell of the isotonic lever together with the first derivative of the latter signal, were displayed on Tectronix 502 A Oscilloscope. The differentiation wa achieved by means of an RC-circuit, time constant 0.5 msec.

The temperature wa kept constant at 30 ± 0.5 C in all experiments.

Solutio

The Ringer solution used was of the following composition (mM): NaCl 120, NaHCO₃ 25, KCl 5, CaCl₂ 2.0, NaH PO 1.5, MgSO 1.5, Dextrose 10. All hemicals were of analytical grade and glass distilled w ter was used. The solution w bubbled w th 95 % O₂ and 5 % CO₂. The pH of the solution was 7.4.

Memb   potent al m su ement

Microelectrodes w th resistance of 10—20 MΩ were used. They were filled with 3 M KCl by boiling under reduced pressure. The recording arrangement onsisted of cathode follower input stage and Tectronix 502 A Oscilloscope. The input capacitanc w approximately 2.5 pF.

Results

Relat n b twee isom t c tension and muscle length

The length dependence of the resting and the active tension in a human foetal papillary muscle is illustrated in Fig. 1. This relation is similar to that found in corresponding measurements of papillary muscles of the cat (Spiro and Sonnenblick 1964). The most striking difference between the tension curves for the my ocardium and the skeletal muscle is the relatively high resting tension necessary for attaining maximal acti e tension in the heart. The following experiments were designed to in estigate changes in active tate in association with alterations in muscle length, and to find out whether alterations in active state could be correlated with any variation in ction potential duration. In order to minimize influence of parallel elasticity the experiments were performed at a low resting tension, 100—200 dyn.

The time cou se of the act ve state i elation to muscle length

The time course of the active tate was determined according to the approach devised by Jewell and Wilkie (1960) and used previously in studies of rabbit (Edman, Nilsson and Grieve 1966, Edman and Nilsson 1968) and cat (Sonnenblick 1967) papillary muscles. After n initial isometric phase the upper end of the preparation was released at a preset moment after the stimulus to enable isotonic shortening of the muscle against a small load (200 dyn). The subsequent shortening occurred in two steps (Edman and Nilsson 1968 Fig. 1). The velocity of shortening during the second, slow phase, considered t represent the movement of the con-

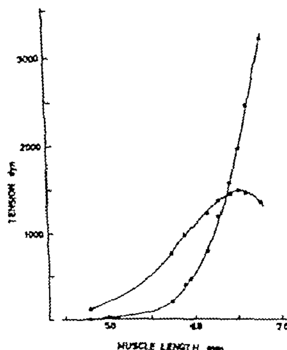


Fig. 1 Length-tension diagram for human fetal papillary muscle. resting tension ○ active tension. Stimulation frequency 36 per min. Largest diameter of preparation 1.2 mm.

tractile unit alone, was used as an index of the degree of activity of this element. The time course of the active state was obtained by releasing the muscle at different moments after the stimulus The velocity measurements used to derive the active state curve were taken at a given amount of shortening of the contractile unit in a series of quick release records. The velocity data so obtained plotted against time after stimulation provide an index of the course of the active state at a constant contractile element length (Edman and Nilsson 1968)

The shape of the active state curve for the human foetal papillary muscle is similar to that for the adult rabbit and at papillary muscles (Brady 1966 Sonnenblick 1967 Edman and Nilsson 1968) As pointed out in these studies, the onset of activ is slow and the activ tate curve has no distinct plateau. In the rabbit papil lary muscle both the intensity and the duration of the active state was shown to be critically dependent on muscle length (Edman, Grieve and Nilsson 1966) This was also found to be the case for the human foetal papillary muscle (Fig) The velocit measurements in one active state curve wer made at a given length of the activ unit f above) In order to study the influence of muscle length upon the active tate the active state curves were constructed f om velocity measurements taken at ious degrees of shortening of the contractile unit (93 5—98 pe cent of resting muscle length) using the same quick-release records n all ases A typical experiment is illu trated in Fig and T bl I ummarizes the re lts obtained in 4 preparations As is evident shorten ng of the contractile unit caused a n the amplitude of the active state curve, an earlier occurrence of maxi

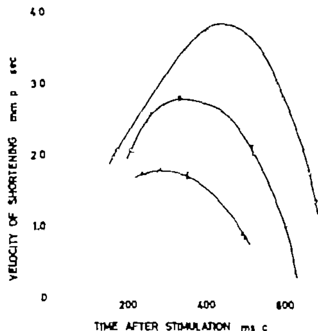


Fig 2. Activ stat curves t three different muscle lengths measured from same isotonic shortening curves after quick releases. Muscle lengths t active stat measurement O 97 0 _ 93 8 and Δ 93.5 per cent of resting muscle length. Preload 700 dyn. stimulation frequency 24 pe min. Dimensions of muscle Length 5 7 mm, largest diameter 0 8 mm (Exp. no. 1 in Table 1

and an earlier disappearance of the acti e state. By 2.9—3 6 per cent shortening of the contractile unit the time from stimulus to 50 per cent decline of the active ate was reduced by 81—155 msec at the temperature and contraction frequency sidered.

Variation of the lati n between the time courses of the active state and the action potential with muscl l ngth

It was considered of nterest to find out whether the length dependence of the duration of the mechanical activity was related to a change of the electrical acti ity As the preparation was mounted according to the requirements of the mechanical recordings, some una oidable movements of the papillary muscle occurred during the transmembrane potential measurements. This might have introduced some error in the determination of the amplitude of the action potential. (In th experiments described in this section the resting membrane potential varied between 56 and 80 mV) However the ariation of the resting membrane potential did not affect the *duration* of the action potential under the present experimental conditions. This is demonstrated in Table II where the variation in action potential duration and resting membrane potential is shown in 8 successive measurements on the same preparation. Table III shows the duration of the action potential obtained with different lengths of muscle from the same preparations as in Table I As is evident, change in muscle length induced no significant change in the time course of the action potential within the range tudied. It may thus be concluded that the shortening of the duration of the acti e state produced by shortening of the contractile unit is not due to any alteration of the time course of the action potential.

TABLE I

Exp. no.	Muscle length, per cent of resting length	Max. active state intensity relative value	Time from stimulus to attainment of 90 per cent of max. activity msec	Time from stimulus to 50 per cent decline of active state msec
1	97.0	1.00	233	653
	95.8	0.72	305	578
	93.5	0.46	—	498
2	96.3	1.00	252	550
	95.1	0.74	232	478
	93.3	0.52	218	432
3	98.2	1.00	330	680
	96.5	0.55	296	624
	95.4	0.39	228	540
4	97.2	1.00	230	465
	95.8	0.75	215	435
	94.4	0.61	—	384

Resting tension 100 dyn in exp. no. 3, 200 dyn in other experiments. Isotonic load in active state measurements 200 dyn in all experiments. Stimulation frequency 24 per min in exp. no. 1 and 2, 36 per min in no. 3 and 4.

TABLE II

Membrane potential, mV	Time from stimulus to half decay of action potential, msec	Deviation of action potential duration from mean, per cent
56	496	+0.8
58	484	−1.6
65	508	+3.2
65	488	−1.6
65	472	−4.1
71	504	+2.4
71	488	−0.8
78	496	+0.8
	Mean ± SEM 492 ± 4.4	

Influence of stimulation frequency on the active state

In ventricles from most mammalian species increased stimulation frequency exerts a positive inotropic influence (Koch Weser and Blinks 1963). This is true also for the human foetal papillary muscle as shown in Fig. 3. The experiments to be described in this section were undertaken to elucidate changes in active state and action potential duration associated with this inotropic intervention. Fig. 4 shows action potentials, isometric twitch responses and active state curves recorded under

TABLE III

Exp. no.	Muscl length, per cent of resting length	Duration of action potential, msec	Number of measurements
1	100 0	600 (580—620)	3
	9 8	626 (624—628)	2
	93.5	610 (600—620)	2
2	100.0	511 (500—522)	2
	9 8	503 (491—506)	3
	93 1	48((480—492)	2
3	100 0	664 (6 6—672)	2
	98.(	670	1
	9(4	664	1
4	100 0	440 (440—440)	2
	97.0	440 (428—460)	3
	91.0	454 (45 —456)	2

Same experiment as i T ble I

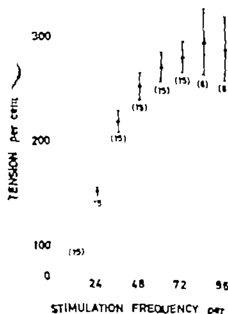


Fig 3 Relation between frequency of contraction and tension (±SEM) Tension at stimulation frequency 12 per min is taken as 100 *per cent for ach preparation.* The bsolute values of peak isometric twitch tension at stimulation rate 1 per min 103—691 dyn (no corrections made for differences in muscle diameter) Numbers in brackets indic t number of preparations.

identical conditions in one pap llary muscle at stimulation frequencies of 36 and 7? min. As can be seen, increased ontraction frequency caused an increase in the intensity of the active state, an earlier occurrence of maximum and a more rapid decline of the activity In 5 expts. of t is kind th isometric twitch response increased by ?0 per cent (range 12—28 per cent owing to the altered pacing of the muscle

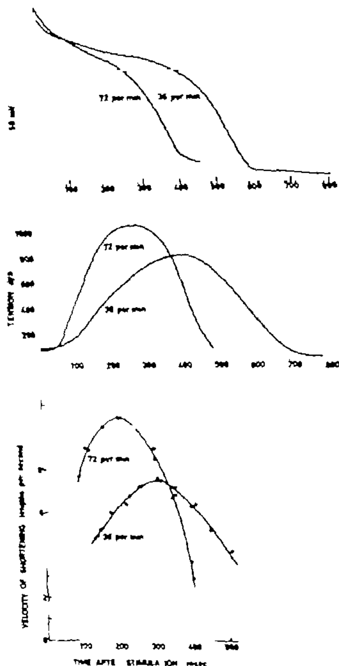


Fig. 4. Action potentials, isometric twitches and active state curves at contraction rates 36 and 72 per min. (Due to different time scales on oscilloscope records, the curves have been redrawn. Resting tension 100 dyn. Same preparation as in Fig. 2. In the active state curves, all measurements refer to the same length of the contractile unit.

The corresponding intensification of the active state was 59 per cent (range 39—94 per cent.)

At 36 stimulations/min the active state reached 90 per cent of its maximal value within 216 ± 14 (Mean ± SEM) msec and declined to 50 per cent of that value 510 ± 13 msec after the stimulation. An increase in stimulation frequency to 72/min reduced these values to 125 ± 7 msec and 408 ± 18 msec respectively. There was no difference between the two frequencies studied regarding the time from maximal active state intensity to 50 per cent decline level: it was 201 ± 14 msec at 36 stimulations/min and 206 ± 11 at 72 stimulations/min.

The duration of the action potential at different stimulation frequencies

A change in the rate of stimulation from 36 to 72/min reduced the duration of the action potential, measured at 50 per cent level, from 548 ± 40 to 352 ± 16 msec. The mean shortening of the action potential duration in consequence of the increased stimulation frequency was thus considerably greater than the reduction of the active state measured at the same level. It was difficult to obtain active state curves at stimulation frequencies below 36/min due to the low tension produced by the muscle under these conditions. However, in some preparations measurements could be made at 12, 24 and 36 contractions/min with results similar to those reported above, i.e. increasing stimulation frequency causing a greater reduction in the duration to 50 per cent decline level of the action potential than of the active state.

Discussion

This investigation deals with active state measurements in papillary muscles from human foetuses and the correlation between the time course of the active state and the duration of the action potential. The papillary muscles were obtained from foetuses of an embryological period when the organogenesis is considered to be complete (Hamilton, Boyd and Mossman 1964). The active state curves were obtained by determining the shortening velocity of the contractile element following a quick release (Edman and Nilsson 1968). All velocity measurements forming the basis of a given active state curve refer to the same length of the contractile unit. This has two advantages: first the influence of muscle length itself on the active state can be adequately investigated and, second, this influence can be controlled during the study of other variables, e.g. contraction rate.

The time from stimulus to half decay of the active state is progressively reduced when the velocity measurements are performed at shorter muscle lengths. Similar conclusions have been reached for rabbit papillary muscle (Edman and Nilsson 1968) and skeletal muscle (Edman and Kiessling 1966) concerning the degree of extension of the muscle cell as a determinant of the time course of mechanical activity. Changes in muscle length did not alter the duration of the action potential in the present investigation. This is in accordance with the results of Dudel and Trautwein (1954) who investigated the influence of resting muscle length on ac-

tion potentials of cat papillary muscles. It appears reasonable to assume that the intensity of the active state at a given length of the preparation reflects the available concentration of some limiting activator substance, probably Ca or a Ca-complex (Edman, Grieve and Nilsson 1966 Edman and Nilsson 1968) One probable explanation for the reduction of the duration of the active state with decreasing muscle length is an increased rate of inactivation of this hypothetical activator although a change in the release mechanism cannot by excluded. It is not clear however whether differences in length of the active unit are the only basis for the changes in the active state, or whether the movement *per se* of this unit also influences the mechanical activity

An increase of the contraction frequency of the preparation augmented the isometric twitch tension This relation in fetal papillary muscle is similar to the general frequency dependence pattern described for most mammalian species studied (Koch-Weser and Blinks 1963) In the cat papillary muscle at 37° C the twitch tension is highest at a higher contraction frequency (about 120 per min) (Koch Weser 1963) than reported here (84 per min)

The discrepancy might, in part, be plained by the relatively low temperature used in the present experiments, 30 C. (The temperature was hosen because it llowed sufficient time resolution of the mechanical recordings.) Low temperature in itself has a positiv inotropic effect and, thereby increases the oxygen requirement and the tendency t tissue hypoxia (Kaufmann and Fleckenstein 1965) F rthermore the diameters of some of the preparations used exceeded that believed to be critical for oxygen diffusion in isolated cardiac tissue (Cranefield and Greenspan 1960 Koch-Weser 1963) This might hav shifted the optimum tension also tow rds lower stimulation frequency by causing central hypoxic core in the preparation which might be larger at higher stimulation frequency All activ state measurements were, however carried ou during steady state conditions, as judged from constant isometric tension.

A change in stimulation frequency from 36 to 7 per min increased the active tate intensity by approximately 60 per cent and shifted the active state curve towards stimulus. It should be observed that the whole active state curve was shifted towards the stimulus, although the time from maximal active state intensity to half decay level remained unaltered. If the active state curve reflects the concentration of an intracellular activator the results would be in line with the idea that the release of the activator agent occurs at a higher rate, that t is initiated earlier after timulus and maintained for a shorter period of time at the higher stimulation frequency F rthermore, the inactiv tion mechanism would seem to proceed according to the same first order reaction at both frequencies studied (*cf* Niedergerke 1963 a, b)

The results showed that when the contraction frequency is increased the d ration of the action potential decreases relatively more than that of the active state This is n accordance with the findings of Trautwein and Dudel (1954) who investigated hanges in action potential and isometric tw tch tension induced by altered contraction frequency According to their results the action potential duration was consistently found to be reduced to a greater extent than the time from stimulus t peak twitch tension.

There are reasons to assume that the time of the depolarization beyond a giv

value is a determinant for the time course of the mechanical activity (Edman, Griewe and Nilsson 1966) One possible explanation of the apparent nonparallel changes of the duration of the electrical and mechanical activities might be a lowering of the mechanical threshold at the higher stimulation frequency This would maintain the release of activator substance for a relatively longer period during the course of an action potential and thereby prolong the duration of the active state relative to the action potential duration.

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Acta physiol. scand. 1968 73 54—57

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The Effect of Prostaglandin E_1 on the Response of the Rabbit Oviduct to Hypogastric Nerve Stimulation

By

JAN BRUNDIN

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Abstract

BRUNDIN, J *The effect of prostaglandin E on the response of the rabbit oviduct to hypogastric nerve stimulation* Acta physiol. scand. 1968. 73 54—57

The effect of sympathetic nerve stimulation was studied on the response of rabbit oviducts under the influence of prostaglandin E (PGE₁) PGE₁ inhibited the constrictory response to nerve stimulation and injection of noradrenaline (NA) A possible physiological role of PGE is discussed

The prostaglandins of the E series decrease the resistance to perfusion through the uterine part of the rabbit oviduct (Horton, Main and Thompson 1963) The uterine half of this organ — the isthmus — is richly innervated by noradrenergic nerves from the hypogastric plexus (Brundin 1964) Supramaximal electrical stimulation of these nerves elicits an obliteration of the lumen of the organ by contraction of the circular muscles (Brundin 1965) The present study reports evidence for an inhibition of this α-receptor response by the administration of the prostaglandin E

Material and methods

Oophorectomized rabbits pretreated with atropine and heparine were used. Under general anesthesia (pentobarbital) a polyethylene catheter was inserted in the lumen of the isthmus from the uterine end The resistance to constant perfusion flow (0.9 % NaCl-solution, 16 or 28 µl/min) was recorded as well as the systemic arterial blood pressure. The distal end of the transected hypogastric nerve was stimulated electrically below the inferior mesenteric ganglia. The test solutions, prostaglandin E (PGE and noradrenaline (NA) were administered through an ear vein. A detailed description of the experimental procedure has been presented previously (Brundin 1965)

Results

The pressure curve recorded during perfusion of the organ and stimulation of the hypogastric nerve is presented in Fig 1 The rate of rise of the perfusion pressure curve, seen in the figure, indicates occlusion of the lumen of the isthmus at the start

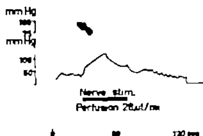


Fig 1 T p traci g systemic arterial blood pressure Note decrease during hypogastric nerv stimulation (17.5 p/sec) *Lower tracing* oviduct perfusion pressure. H pogastric nerve stimulation Elicits oviduct occlusion Dotted line occlusion type of pressure rise obtained w th tip of perfusion catheter clamped.

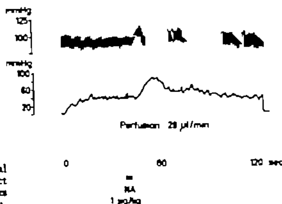


Fig. 2. *T p tracing* systemic rterial blood pressure. *Lower traci g* oviduct perfusion pressure. Noradrenaline causes occlusion of oviduct. Dotted line occlusion type of pressure rise.

of the infusion. Th passage of fluid through the lumen began at 40 mm Hg The occlusion of the oviduct reappeared (*cf* dotted line) during the hypogastric nerve stimulation. Simultaneously a further decrease of the blood pressure occurred. Fig 2 is a record of the occlusion of the isthmus after a moderate dose of NA. Th administration of PGE (Fig 3) reduced the perfusion pressure and abolished the spon taneous pressure fluctuations. The arterial blood pressure decreased promptly The response to nerve stimulation was diminished even 30 sec after the dministration of PGE No occlusion type of the perfusion pressure curve was obtained at that time. Fig 4 shows that PGE also reduced the contraction, normally elicited by NA. At 2 min fter the administration of PGE the blood pressure had returned to normal but the response to hypogastric nerve stimulation was still weak, while the blood pressure fell harply

Discussion

E idendl the intravenous injection of NA failed to cause any marked obl teration of the l men of the isthmus under the influence of PGE suggesting that th inhibition caused by PGE cted on the effector side of this nerve-smooth muscle ystem This inh bition of the muscle response was pronounced enough to accou t for all of the inhibitory effect. The reduced effect of nerv stimulation after PGE appeared

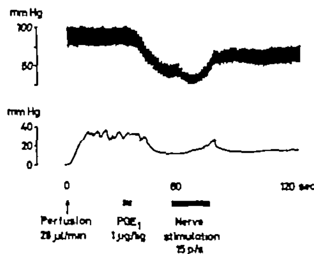


Fig. 3. Top tracing: systemic arterial blood pressure. Lower tracing: oviduct perfusion pressure. After prostaglandin E no spontaneous oviduct activity, decreased perfusion pressure and blood pressure. Note absence of occlusion type of perfusion pressure response and further decrease of blood pressure during hypogastric nerve stimulation.

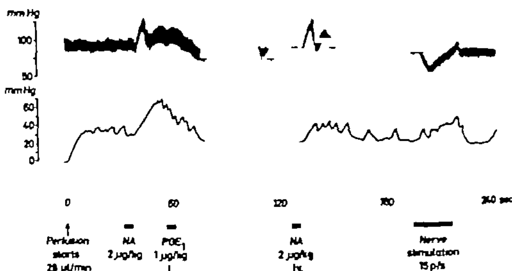


Fig. 4. Top tracing: systemic arterial blood pressure. Note reduction during hypogastric nerve stimulation. Lower tracing: oviduct perfusion pressure. After prostaglandin E diminished response to noradrenaline and no occlusion type of perfusion pressure during hypogastric nerve stimulation.

even after that time when the perfusion pressure and the arterial blood pressure had returned to normal values. This implies that an inhibition of the noradrenergic response was still present at a low systemic concentration of PGE. Thus, *e.g.* the passage of sperms through the sphincter of the isthmus might be facilitated even by low concentration of PGE_1 in the seminal fluid.

A direct *in vitro* inhibitory effect of PGE on the response to added NA has been reported for various organs. This effect has been found in the vas deferens, colon, fundic strip and uterus of the rat and in the uterus and tracheal chain of the guinea-

pig (Clegg 1966) On the other hand, PGE_1 causes potentiation of the response to NA in the isolated seminal vesicle of the guinea-pig (Eliasson and Risley 1966) The present results show that PGE_1 also is capable of modulating the response of the rabbit oviduct to noradrenergic nerve impulses *in vivo*

It has recently been described that stimulation of the noradrenergic nerves to the dog spleen causes a release of prostaglandin E_2 from this organ (Davies Horton and Withrington 1967) By analogy a similar release of prostaglandins from the genital organs is conceivable during stimulation of the hypogastric nerves. The decrease of the blood pressure, observed during the nerve stimulation would be compatible with such an assumption. Finally the present results show that PGE_1 modulates th re sponse of the rabbit oviduct to hypogastric nerve stimulation.

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Acta physiol. scand. 1968. 73 58—61

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Potentiation of Tyramine Effect on the Isolated Iris Muscle by Cocaine

By

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Abstract

Lagercrantz, H. *Potentiation of tyramine effect on the isolated iris muscle by cocaine.* Acta physiol. scand. 1968. 73 58—61

Effects of noradrenaline, tyramine and cocaine have been studied on the sphincter of the isolated bovine iris muscle preparation. After administration of cocaine in low concentrations ($0.5 \times 10^{[illegible]} - 2 \times 10^{[illegible]}$ M) applied for 2—10 min the tyramine response was increased in 5 cases out of 12. Various explanations for this effect of cocaine are discussed.

The finding of Tainter and Chang (1927) that cocaine antagonizes the biological effect of tyramine has later been thought to be due to prevention of *its releasing effect* on catecholamines from their storage sites (Fleckenstein and Stockle 1955). According to Trendelenburg (1961) cocaine is a competitive antagonist *to* tyramine. On isolated adrenergic nerve granules Euler and Lishajko (1965) found that cocaine in a concentration of $10^{[illegible]}$ M has only a *negligible inhibitory effect* on the enhancing action of tyramine on the noradrenaline release, suggesting that the effect of cocaine was on the axon membrane rather than on the storage granules.

In the present study the [illegible] of tyramine on the iris muscle from the cow was studied in [illegible] the presence of low concentrations of cocaine.

Methods

Isolated iris muscles from cows were used as described by Djahanguiri (1965) and Jakobs (1964). The eyes were obtained from the slaughter-house and kept in ice-cold Tyrode solution until they were prepared within a few hours. About one cm of the peripupillary part of the iris was excised and fixed with threads which were suspended between a platinum hook and a strain gauge for isometric tension recording. The tension of the muscle was adjusted to a level corresponding to about 400 [illegible]. The hook was mounted in a 4 ml chamber which was filled with Tyrode solution (NaCl 0.8 %, $NaHCO_3$ 0.1 %, KCl 0.2 %, $CaCl_2$ 0.02 %, $MgCl_2$ 0.02 %, NaH_2PO_4 0.0[illegible] %, glucose 0.1 %, pH = 7.4). The bathing fluid was aerated with 5 % CO_2 in O_2. The bath temperature was 37° C. The drugs were injected directly into the chamber. Before each addition of the drug the chamber was rinsed for 1— [illegible] min with Tyrode solution at a rate of [illegible] 0 ml/min. Atropine was added in a concentration of $10^{[illegible]}$ M. The following drugs were used: Noradrenaline bitartrate, tyramine hydrochloride and cocaine hydrochloride. Concentrations are given as the base.

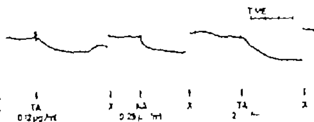


Fig. 1 Effect of 0.12 μg/ml tyramine on the iris muscle before and after pretreatment with noradrenaline for 1/2 min in a concentration of 0.25 μg/ml. Preparation washed t X



Fig. 2. Effect of 0.25 μg/ml tyramine before (A) and fter (B) cocaine incubation during 15 min i a concentration of 0.5 × 10 M

Results

Noradrenaline in a low concentration (0.25—0.5 μg/ml) always caused relaxation of the iris muscle preparation. A higher dose (1—2 μg/ml) caused relaxation in 5 cases as well, but in the remaining 4 cases a contraction was noted. In these cases the iris muscles seemed to be less reactive to noradrenaline and tyramine in low concentrations. The contraction was readily blocked by the alfa-blocker phenoxybenzamine (25 μg/ml)

Tyramine also regularly produced a relaxation, which developed more slowly than that produced by noradrenaline Tachyphylaxis was not observed after repeated doses Thus 6 successive doses with times between each dose produced nearly the same response. In some cases the response to tyramine after noradrenaline administration and subsequent washing was increased (Fig 1)

Cocaine tself sometimes produced a small and slow relaxation of the iris muscle preparation The response to tyramine was regularly inhibited by cocaine in a concentration of 3 10 ^{5} M applied for 10 min, while lower concentrations of cocaine, 0.5×10 — 10 M, pplied for 2—10 min, in 5 cases out of 12 caused a potentiation of the t ramine response (Fig) In the remaining cases no consistent effect of ocaine on the tyramine response could be observed.

Discussion

Th fall n tensi n of the iris muscle preparation observed after addition of noradrenaline to the bath is caused by relaxation of the sphincter muscle innervated by adrenergic nerves ccording to Takáts (1964) and Schaeppi and Koella (1964) While the iri phincter of the cat contracted after a small dose of noradrenaline nd rela ed after a higher dose in the experiments of the latter authors the cow iris n the present experiments beha ed in the opposite wa relaxing w th low doses and often ontracting with higher ones. Accord ng to Schaeppi and Koella (1964)

the alfa receptors, mediating contraction, are spread in the muscle while the beta-receptors are situated near the nerve endings. This is in agreement with our observation that tyramine consistently produces relaxation.

The increased response to tyramine observed after noradrenaline administration (Fig 1) is probably caused by an increased uptake of noradrenaline subsequently released by tyramine. It is conceivable that the granules in some cases have been partially depleted in the isolated preparations, causing a weaker response which can be restituted after uptake of noradrenaline.

Our observation that the effect of tyramine may be potentiated by cocaine suggests that it is due to an enhancement of the noradrenaline action. The usually observed effects of cocaine on the actions of noradrenaline and tyramine—enhancing and inhibitory respectively—are commonly explained by assuming that cocaine inhibits the uptake of the amines through the axon membrane (Macmillan 1959 Whitby Hertting and Axelrod 1960 Muscholl 1961).

A potentiating action of small doses of cocaine on the effect of tyramine has earlier been reported on the nictitating membrane (Holtz, Oswald and Stock 1960 Trendelenburg 1961). According to Holtz *et al.* the most likely explanation is that the small dose of cocaine does not suffice to inhibit the amine releasing activity of tyramine but still can potentiate the endogenously released noradrenaline. The results reported in the present paper seem to be adequately explained in the same way. The potentiating effect of cocaine on the tyramine response in the experiments by Holtz *et al* and Trendelenburg was obtained on an organ supplied predominantly with α-receptors. Our results have given evidence that the same kind effect is also obtained on β-receptors.

In addition to the possible explanation mentioned by Holtz *et al* it seems reasonable to consider the inhibiting effect by tyramine on noradrenaline reuptake (Burgen and Iversen 1965). According to Iversen (1967) the inhibitory effect of tyramine is chiefly responsible for its action, since very small amounts of noradrenaline are released by tyramine. It is also known that cocaine is a competitive antagonist of tyramine (Trendelenburg 1961). It might therefore be assumed that the direct amine releasing activity of tyramine (Euler and Lishajko 1968) is not inhibited to any greater extent by small doses of cocaine and that the recapture of the released noradrenaline is further reduced because of the combined effect of tyramine and cocaine at the membrane sites. This may also apply to non-specific uptake sites (Brown 1965). However the possibility that cocaine sensitizes the target cells of the effector organ by alteration of receptors, as suggested by Maxwell *et al.* (1959) or otherwise, cannot be excluded. The sympathomimetic action observed with cocaine itself in low concentrations (Campos, Stitzel and Shideman 1963) also observed in our experiments, may be explained in a similar way assuming a small leakage of the transmitter from the nerve endings the effect of which is potentiated by cocaine.

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Acta physiol. scand. 1968. 73. 62—74

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Studies on Uptake and Decarboxylation of Histidine by Isolated Rat Mast Cells

By

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Received 5 October 1967

Abstract

Cabut, M. and Ö. Haegermark. *Studies on uptake and decarboxylation of histidine by isolated rat mast cells.* Acta physiol. scand. 1968. 73. 62—74.

Isolated rat mast cells, when incubated in a medium containing histidine-H³, took up and decarboxylated histidine, thus forming histamine. Uptake and decarboxylation were measured as a function of incubation time and extracellular histidine concentration. The influence of amino acids, amines, inhibitors of cell metabolism, and inhibitors of histidine decarboxylase was studied on these two processes. It was concluded that at least part of the histidine transport into the cell was mediated by a carrier mechanism. In the presence of the histidine decarboxylase inhibitor NSD-1055 the amount of histidine accumulated by the cells reached a maximum level within 10 min and was not increased significantly by prolonging the incubation. The Histamine-H³ formed by the mast cells *in vitro* from histidine-H³ was bound to granules of osmically disintegrated cells, but to a lesser extent than that originally present in the cells. Only a very small proportion of the histidine taken up in the presence of a decarboxylase inhibitor was recovered in the granular fraction. Treatment of mast cells with compound 48/80 released the same relative amounts of the histamine formed *in vitro* and of that originally present in the cells.

Mast cells of rat peritoneal cavity have the capacity to take up and decarboxylate histidine, thus forming histamine which is then stored in the cells (Schayer 1956). Similar processes have been demonstrated in mast cells from other sources, *e.g.* from mastocytoma in mouse (Day and Green 1962a) and dog (Lindell, Rorsman and Westling 1959) and from urticaria pigmentosa in man (Birt, Hagen and Zebrowski 1961, Lindell, Rorsman and Westling 1961). Mast cells from mouse mastocytoma and rat peritoneal cavity are also able to concentrate extracellular histamine (Day and Green 1962b, F[illegible] and Green 1964), a process which seems to occur through passive diffusion into the cells and subsequent binding to the intracellular granules (Day and Stockbridge 1964, Cabut and Haegermark 1966). Although the decarboxylation of histidine has been studied in mast cells from the rat (Rothschild and Schayer 1959) and from mouse mastocytoma (Weissbach, Lovenberg and

[1] Visiting scientist Nov. 1962—Dec. 1964.

Udenfriend 1961 Aures and Clark 1964) little is known about the mechanism of its transport into the cell. There is much evidence, however that amino acids in general are transported by "carrier" mechanisms through the membranes of a great variety of cells (for ref. see Holden 1962)

The aim of the present investigation was to study histidine uptake by isolated mast cells and the relation between the uptake and the decarboxylation process. A comparison was also made between the histamine synthesized *in vivo* and that originally present in the cells with respect to the intracellular localization and the release.

Methods

Cell preparation

Pleural and peritoneal mast cells were isolated from male Sprague-Dawley rats (300—500 g) by density gradient centrifugation in Ficoll (Thon and Uvnäs 1966) After isolation, the cells were pooled and washed once with a buffered salt solution containing NaCl, 154 mM KCl, 2.7 mM $CaCl_2$, 0.9 mM human serum albumin, 1 mg/ml buffered with 10 per cent (v/v) Sorensen phosphate buffer ($Na_2HPO_4 + KH_2PO_4$ 67 mM) pH 7 After resuspension in fresh salt solution, the cells were counted in Bürker chamber and then incubated. Usually —3 million mast cells were obtained from each rat.

Incubation technique

Incubations were run in 15 ml centrifuge tubes in final volume of 0.5 ml. To each tube 0.4 ml of the cell suspension was added and incubated at 37 C for 10 min prior to the addition of 0.1 ml (as a rule 10 μCi) of histidine-H^3 solution. When the influence of inhibitors was studied, the cells were preincubated with inhibitor at 37 C for 30 min, and the histidine-H^3 was then added. All incubations were carried out in duplicate The number of cells in each tube varied in different experiments between 0.3×10^6 and 0.8×10^6

The same technique as previously described (Cabut and Haegermark 1966) was used for terminating the incubation and washing the cells, with the difference that the solutions were kept ice-cold also during the washings. The level of radioactivity in the last washing did not significantly exceed the background. Extracts were prepared from the washed cells by suspending them in 1 ml of 0.1 N HCl and heating in boiling water bath for 5 min. Aliquots of the extracts were taken for measurement of radioactivity and histamine and, in some experiments, for chromatography

Analysis

Radioactivity was determined by liquid scintillation counting as described previously (Cabut and Haegermark 1966)

Histamine was assayed by fluorometry after condensation with o-phthalaldehyde (OPT) according to the method of Shore, Burkhalter and Cohn (1959) but the purification step was omitted and OPT was added directly to the alkalinized samples (Fredholm and Haegermark 1967)

In some samples histamine-H^3 formation was estimated by paper chromatography Aliquots for determination of radioactivity and histamine were first removed. Next, the remaining cell extract (≈ 0.5 ml) was freeze-dried, re-dissolved in small volume (≈ 20 μl) of distilled water and applied to Whatman No. 1 paper Ascending chromatography was performed with n-butanol/pyridine/water (1 1 1) for separation of histidine and histamine (Smith 1960) Radioactivity of the dried chromatograms was measured by cutting the paper into 1-cm strips and immersing these in the scintillation fluid without previous extraction. Virtually all the radioactivity was recovered in peaks corresponding to the histamine and histidine spots on the non-labelled reference stained with ninhydrin. However in extracts from cells which had been incubated for 160 min, a small peak constituting about 5 per cent of the total radioactivity of the chromatogram appeared at the starting point of the chromatogram (cf Discussion) The histamine-H^3 formed from the histidine-H^3 taken up is expressed as percentage of the total radioactivity of the chromatogram.

Materials

L-Histidine-H^3 specific activity 1.1 Ci/mmole (Schwarz Bio Research, Inc., Orangeburg, N.Y. USA) was used in the initial experiments.

L-Histidine-2,5-(ring) H^3 specific activity 11.1—11.9 Ci/mmole (The Radiochemical Centre Amersham, England). Before use, the histidine-H^3 was diluted with unlabelled histidine to adjust the specific activity to 10 μ Ci/mmole.

Ficoll (AB Pharmacia, Uppsala, Sweden)

Human serum albumin (AB Kabi, Strängnäs, Sweden)

Compound 48/80 (AB Leo, Hälsingborg, Sweden)

L-Tryptophan and 5-Hydroxy L-tryptophan (Sigma Chemical Co., St. Louis, Mo., USA)

4 Bromo-3-hydroxy benzyl oxyamine-dihydrogen-phosphate (NSD-1055) (Smith and Nephew Ltd., Harlow, England)

D-2 Hydrazino-3-[4(5)-imidazole] propionic acid hydrochloride (-hydrazino analogue of histidine α-HH) Merck, Sharp and Dohme Lab., New York, USA.

Other substances were obtained from normal commercial sources.

Results

In the present work the term "histidine uptake" means the total radioactivity present in the cell extracts and the term "histamine formation" is used to denote the percentage of this radioactivity which is due to histamine-H^3.

Time course of histidine uptake and histamine formation

Histidine when present at an extracellular concentration of 2 μM was taken up by the mast cells at 37° C. This process was still proceeding after 160 min, although the rate decreased progressively during the incubation (Fig. 1). The concentration of extracellular histidine was virtually constant during the incubation as less than per cent of the histidine added to the medium was accumulated by the cells during 160 min.

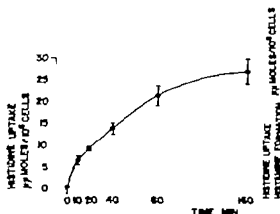


Fig. 1

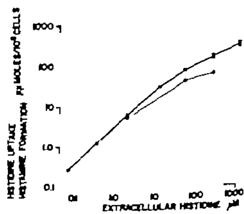


Fig. 2

Fig. 1. Time course of histidine uptake. Extracellular histidine-H^3 concentration, 2 μM. Each point represents the mean value of 3 expts., vertical bars indicate S.E.

Fig. 2. Histidine uptake and histamine formation during 10 min as a function of extracellular histidine concentration. Each point, ●——● represents the mean uptake of 4 expts. S.E.< ± 10 %, except where indicated with vertical bars. Multiplication of these values by the percentages of histamine formed, shown in upper part of Table IV gives the absolute amounts of histamine formed, ○– – –○

TABLE I Histamine formation as function of incubation time. Extracellular histidine concentration, 2 μM

Incubation time, min	Number of expts.	Histamine formed, as a per centage of histidine taken up mean and range*
10	2	81 (79—82)
20	2	86 (83—89)
40	1	87
80	1	93
160	2	93 (90—96)

The figures are taken from the experiments show in Fig 4a and 4b.

TABLE II Ratio of intracellula /extracellular radioactivity concentrations as function of incubation time nd extracellular histidine concentration. For comparison, right part of tabl contains results obtained when mast cells were incubated in histamine-H^3 (Cabut and Haegermark 1966) Concentration ratio = cpm of 10^6 cells/cpm of 1 μl of medium

Incuba ion time, min	Incubation in histidine H^3			Incubation in histamine-H^3		
	Extra cellular histidine μM	Number of expts	Conc. ratio mean ± S.E	Extracellular histamine μM	Number of expts	Conc ratio mea ± S E.
10	2	3*	3.7 ± 0 3			
20	2	3	5.3 ± 0.3			
40		3	7 9 ± 0.2			
60	9 1	7	16.1 ± 1.3	10	6	2.8 ± 0.2
80	2	3*	12.2 ± 0.3			
160	2	3	15.3 ± 0.5			
160	5.7	19	22.8 ± 1 4	12.5	9	4 7 ± 0.7

The same experiments as are show in F g 1

In some samples the rate of histamine formation was also studied (Table I) After 10 min, about 80 per cent of the histidine taken p was converted nto histamine and after 160 min more than 90 per cent of the cellular radioacti t was due to histamine H^3

The ratio of intracellular to extracellular radioactiv ty concentrations was calculated by di iding the counting rat (in cpm) of 10^6 cells, the volume of which pproximately equals 1 l, by the counting rate of 1 μl of medium In T ble II the ratios corresponding to the points n Fig 1 are given, together w th th values ob-

tained from two other series of experiments where higher concentrations of extracellular histidine were used. For comparison the ratios obtained when incubating the cells in histamine (Cabut and Haegermark 1966) are included in Table II. From these figures it can be calculated that the capacity of the mast cells to take up histidine was about 3—6 times higher than for histamine.

Influence of extracellular histidine concentrations on histidine uptake and histamine formation

Histidine uptake during 10 min was measured as a function of extracellular histidine concentration (Fig. 2). When the extracellular histidine concentration was raised 5-fold over the low concentration range it resulted in an almost 5-fold increase in histidine uptake while the same increment at the highest concentrations only led to a 2 [?] fold increase in uptake. No saturation was reached, although the histidine concentration was raised 15,000-fold.

The percentage of histidine H taken up which was converted into histamine H was observed to decrease with increasing extracellular histidine concentrations (Table IV, upper part, without inhibitors). From these figures the absolute amounts of histamine formed were calculated and the results plotted in Fig. 2. The high concentrations of extracellular histidine were reached by addition of increasing amounts of unlabelled histidine. This reduced the specific activity to such an extent that the uptake of labelled histidine at the highest extracellular concentration was too low to allow chromatographic determination of histamine formation.

TABLE III. Influence of amino acids, amines and enzyme inhibitors on histidine uptake during 10 min, expressed as percentage of uptake in controls. Cells were incubated with inhibitor for 30 min prior to addition of histidine H (final concentration, 2 µM). Values are given as means ± S.E. Figures in parenthesis denote number of expts.

Inhibitor	Concentration of inhibitor					
	10[illegible] M	10[illegible] M	10[illegible] M	10[illegible] M	10[illegible] M	10[illegible] M
Tryptophan				87 ± 3(5)	44 ± 7(5)	
5-HTP				78 ± 6(5)	39 ± 2(6)	16 ± 3(3)
Histamine					94 ± 6(4)	86 ± 3(4)
5-HT					86 ± 9(3)	102 ± 6(3)
NSD-1055	55(1)	60 ± 4(1) [?]	54 ± 6(4)	37(1)		
α-HH			94 ± 7(3)	69 ± 8(5)	56 ± 4(3)	
KCN				78 ± 9(4)	53 ± 9(7)	27 ± 3(6)
DNP					64 ± 5(4)	
Iodoacetic acid					106(2)	
DNP + Iodoacetic acid (equal conc.)					58 ± 4(4)	

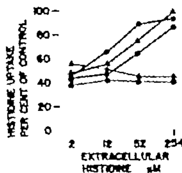


Fig 3 Influence of inhibitors on histidine uptake at various extracellular histidine concentrations. Incubation time, 10 min.
○———○ NSD-1055 10 M (1 expt)
▲———▲ NSD-1055, 10⁻⁴ M (1 expt)
●———● 5-HTP 10 M (2 expts)
▲- - -▲ DNP 10 M + iodoacetic acid, 10 M (1 expt)
●- - -● KCN 10 M (1 expt)

Influence of enzyme inhibitors amino acids and amines on histidine uptake

The influence of various substances which might interfere with transport of histidine into the cell and/or its subsequent decarboxylation, was investigated.

The effect on uptake in cells incubated for 10 min with 2 μM histidine H³ are shown in Table III Inhibition was caused by the amino acids L-5-hydroxytryptophan (5-HTP) and L-tryptophan, precursors of 5-hydroxytryptamine (5-HT) which, like histamine, is synthesized and stored in rat mast cells (Benditt *et al* 1955, Lagunoff and Benditt 1959) Conversely histamine and 5-HT had no inhibitory effect. The two inhibitors of histidine decarboxylase, 4-bromo-3-hydroxybenzyloxyamine (NSD-1055) and the α-hydrazino analogue of histidine (α HH) (Reid and Shepherd 1963, Levine, Sato and Sjoerdsma 1965) in concentrations that completely blocked the histamine formation (see below) reduced the amount of radioactivity accumulated to about half that of the controls.

A marked decrease in uptake was found with potassium cyanide (KCN) which inhibits not only the respiratory enzymes, but also histidine decarboxylase (Dixon and Webb 1964) The uncoupling agent 2,4-dinitrophenol (DNP) caused a reduction of the uptake. The glycolysis inhibitor iodoacetic acid was combined with DNP in an attempt to cause a more effective block of the cellular energy supply but no significant decrease in the uptake beyond that exerted by DNP alone was observed.

A few of these substances, in concentrations giving about 50 per cent inhibition under the above-mentioned conditions, were also studied in combination with varying concentrations of extracellular histidine (Fig. 3) The inhibitory effects of 5-HTP and NSD-1055 were found to be almost reversed by high histidine concentrations, whereas the effects of KCN and DNP + iodoacetic acid were not altered by raising the extracellular histidine concentration.

The influence of DNP + iodoacetic acid and NSD-1055 on the uptake measured as a function of time is illustrated in Fig 4a and 4b.

Influence of inhibitors on histamine formation

Table IV gives a summary of the results obtained in those inhibitor experiments where histamine formation was studied in addition to histidine uptake Incu

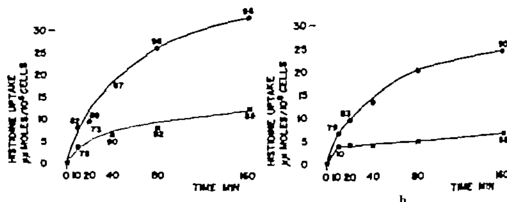


Fig 4 Influence of inhibi ors on histidine uptake as function of incubation time. Extracellular histidine oncentration, 2 μM. The figures t the points denot histamine formed, expressed as percentage of histidine taken up.
) DNP 10 M, + iodoacetic acid, 10 M, ■ – – – ■ Control without inhibitor ● —— ●
b) NSD-1055, 10 M, ■ —— ■ Control without inhibitor ● —— ●

TABLE IV Influence of extracellular histidine concentration nd inhibitors on histamine formation. For comparison, nfluence on histidine uptake in th pertinent experiments is included (cf text) Incubation time 10 mi

Extra-cellula histidine, μM	Inhibitor	Number of expts	Histamine formation, per cent of histidine taken up (mea nd range)	per cent of con rol without inhibitor (mea)	Histidine ptake per cent of control without inhibitor (mea and range)
2	—	7	80 (72—83)	Control	Control
12	—	1	67	Control	Control
52	—	3	55 (48—61)	Control	Control
254	—	3	39 (26—49)	Control	Control
2	5-HTP 10 M	2	60 (56—63)	75	44 (41—46)
12	5-HTP 10- M	1	60	90	48
52	5-HTP 10⁻⁴M	2	48 (46—50)	87	64 (62—66)
254	5-HTP 10 M	2	38 (37—39)	97	85 (74—96)
2	NSD-1055, 10⁻⁴M	1	0	0	57
254	NSD-10 5 10- M	1	0	0	98
2	KCN 10⁻⁴M	1	0	0	38
254	KCN, 10- M	1	0	0	39
2	DNP 10⁻⁴M + iodo-	1	78	98	56
254	aceta e, 10- M	1	53	136	44
2	NSD-1055, 10⁻⁴M	2	0	0	54 (42—66)
2	NSD-1055 10- M	1	7	9	50
2	NSD-1055, 10⁻⁴M	1	23	29	55
2	α-HH, 10⁻⁴M	1	7	9	53
2	α-HH, 10⁻⁴M	1	65	81	68
2	α-HH, 10⁻⁴M	1	83	104	100

time was 10 min. For comparison, Table IV also includes values showing the inhibitory effects on histidine uptake. As these values are the mean results of the chromatographed samples only, they are not identical to those presented in Table III and Fig. 3, but constitute part of the material presented there.

The histidine decarboxylase inhibitors NSD-1055 and α HH, and the cytochrome oxidase inhibitor potassium cyanide caused a complete or a marked inhibition of histamine formation. It is of interest to observe that histidine uptake could take place although histamine formation was completely blocked. In the presence of 5-HTP 10^{-4}M, histamine synthesis was somewhat inhibited when the cells were incubated in low concentration of histidine (? μM)

The effect of inhibitors on histamine formation during various incubation periods, is shown in Fig. 4a and 4b. The figures denoted in these diagrams represent the percentage histidine decarboxylated. It can be seen that the combination of DNP and iodoacetate did not significantly affect histamine formation — although histidine uptake was markedly decreased. NSD-1055 which inhibited histamine formation also led to a marked reduction in the radioactivity accumulated by the cells. Since the concentration of NSD-1055 used, 10^{-7}M, did not completely inhibit histidine decarboxylase, (*cf* Table IV) 10 per cent of the histidine taken up had been converted into histamine after 10 min incubation, and after 160 min incubation 58 per cent was converted.

Subcellular distribution

After incubation in radioactive histidine for 60 min, the mast cells were disintegrated by sonication and then subjected to differential centrifugation as previously described (Cabut and Haegermark 1966, Fredholm and Haegermark 1967). The 350×*g* sediment contained cell debris and some large mast cell granules, the 1,500×*g* sediment consisted mainly of mast cell granules. In the final supernatant the remaining subcellular particles and soluble materials were recovered. The distribution of radioactivity and of total histamine was determined in these fractions (Table V). It was found that comparatively more histamine than radioactive material was recovered in the granular fraction. About 90 per cent of the radioactivity in the cells was histamine (*cf* Table I). The amount of this newly-formed histamine was small compared to that originally present in the cells. Thus, the distribution studies showed that the histamine formed during 60 min incubation was bound to the granules of sonically disintegrated cells in a smaller proportion than that originally present in the cells.

Histidine uptake during 60 min in the presence of NSD-1055, 10^{-4}M was found to be about 20 per cent of that in the control. Fig. 5 illustrates the distribution of radioactivity and histamine between the subcellular particles in such an experiment. Almost all radioactivity taken up in presence of NSD-1055 was recovered in the supernatant and only a few per cent in the granula fraction. This should be compared with the control granules which in this experiment contained 40 per cent of the radioactivity

TABLE V Subcellular distribution of radioactivity and histamine in mast cells after incubation with histidine-H^3 during 60 min. Values are expressed as per cent of total radioactivity and histamine present in the cells

Expt. no.	Extracellular histidine μM	350 × g sediment		1500 × g sediment		Final supernatant	
		Radioact	Histamine	Radioact.	Histamine	Radioact.	Histamine
1	9 1	18.9	17.3	41.5	61 7	39 6	20.9
2	9 1	9 1	14.6	25.5	73.2	65.4	12.2
3	4 0	2.2	4 7	41.3	86.0	56.5	9.3
Mean		10 1	12.2	36.1	73 6	53.8	14 1

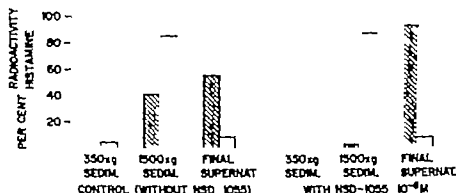


Fig 5. Influence of NSD-1055 on subcellular distribution of radioactivity [] and histamine [], in mast cells incubated with histidine-H^3 4 μM, during 60 min. The columns represent the percentage of total radioactivity and total histamine present in the cells.

Effect of compound 48/80

After incubation with histidine H^3 for 60 min the mast cells were washed three times and then incubated with the histamine liberator compound 48/80 for 10 min at 37 C. The incubation was terminated by centrifuging at 350×g for 3 min Released histamine was recovered in the supernatant. The sediments and supernatants were assayed for both radioactivity and histamine It was found that radioactive material and histamine were released in about the same relative amounts (Fig 6)

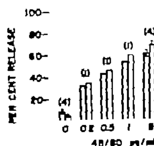


Fig 6 Release of radioactivity [] and histamine, [] from mast cells induced by compound 48/80 The cells were incubated with histidine-H^3 9 1 μM, during 60 min, washed, and incubated with 48/80 during 10 min Each column denotes the mean release, expressed as the percentage of total radioactivity and total histamine present in the cells The figures within brackets indicate the number of expts, vertical bars S.E.

Discussion

The histidine uptake, as measured in the present investigation was actually the sum of several processes: passage of histidine through the cell membrane, decarboxylation of histidine to histamine and binding of the latter in the mast cell granules. Two more processes have to be considered, namely leakage of histamine and histidine from the cells during incubation and washing, and incorporation of histidine into proteins. However only insignificant quantities of labelled histamine could have leaked out during the incubation period, since it was not possible to detect any radioactivity in the histamine peak on paper chromatograms of the incubation fluid. It is difficult to ascertain if radioactive material leaked out when cells were washed to remove the extracellular histidine. Washings were performed at 0° C and there is evidence that passive flows through the membranes of blood platelets are inhibited at low temperatures (Pletscher da Prada and Bartholini 1966) a finding which might also be true for mast cells. Incorporation of histidine into proteins did not contribute significantly to the present results since all the radioactivity of the cell extracts was recovered in the histidine and histamine peaks of the chromatograms, when the cells had been incubated for less than 80 min. Only after 160 min incubation was a small peak observed at the starting point of the chromatograms. This could be due to binding of some of the radioactive material to non-moving molecules, *e.g.* proteins.

One million mast cells contain about 10—20 µg of histamine (/ Moran, Uvnäs and Westerholm 1962, Thon and Uvnäs 1966, Cabut and Haegermark 1966) The amount of histamine formed and stored by 10^6 cells during 10 min incubation in µM histidine solution was about 530 × 10 µg, and in 250 µM histidine the amount was increased 10-fold. Thus it can be calculated that — at an undiminished rate of uptake and histamine formation — it would take about 30 weeks for mast cells surrounded by 2 M histidine to build up a histamine store of 15 µg/10^6 cells. Increasing the extracellular histidine concentration 125-fold would reduce this time to about 3 weeks.

After incubation for 160 min the concentration ratio of *radioactivity* between cells and medium was 15 in one series of experiments and 23 in another. This is of the same order as the figure reported for murine mastocytoma cells 22 after incubation for 120 min (Day and Green 1962a) However in the present experiments the ratio of the concentration of *histidine* in the cells to that in the medium may be lower than 1 as most of the histidine taken up by the cells was converted into histamine. Thus, histidine was not necessarily transported into the cell against a concentration gradient, although the finding that the uptake was reduced by the metabolic inhibitors dinitrophenol and cyanide suggested that energy was utilized in the uptake process. This means that if the uptake was not against a concentration gradient, not active it might be classified as "facilitated diffusion (*cf.* Schanker 1962)

Transport of amino acids through membranes is generally considered to be dependent on carrier mechanisms in the membrane, (*e.g.* Holden 196) Two criteria

indicating the presence of a carrier are saturation kinetics and inhibition by structural analogues and other inhibitors (Wilbrandt and Rosenberg 1961) To assess saturation kinetics the initial rate of the process should be measured. In the present investigation the values obtained after 10 min incubation were regarded as approximately proportional to the initial velocity It was found that the 10 min uptake tended to level off with increasing extracellular histidine concentration. However a definite level of saturation was not reached in these experiments, a finding which might be due to other simultaneous processes, such as diffusion into the cell or unspecific adsorption of histidine. Regarding the second criterion effect of inhibitors, it was found that the structural analogues 5-HTP and tryptophan inhibited the uptake. It should be considered that these agents might compete not only for a common carrier in the cell membrane but also for the intracellular decarboxylase especially as it has been shown that histidine decarboxylase activity of extracts of rat peritoneal mast cells is inhibited by tryptophan and 5-HTP (Rothschild and Schayer 1959) However in the present experiments there is evidence that decarboxylase inhibition could not be entirely responsible for the reduction of the amounts of histidine taken up by the cells in the presence of 5-HTP First, 5-HTP at a concentration causing more than 50 per cent inhibition of the uptake, only reduced the histamine formation to 75 per cent of the control. Second, even complete abolition of the histidine decarboxylase with NSD-1055 or α HH only reduced the uptake to about half that of the control, whereas 5-HTP 10^{-3}M, reduced the uptake to 16 per cent of the control. Thus, it seems justified to conclude that in the present experiments at least of the histidine uptake was mediated by a carrier mechanism, competitively inhibited by tryptophan and 5-HTP Conversely the amines 5-HT and histamine did not influence the uptake significantly

After incubation for 10 min in 2 μM of histidine 80 per cent of the histidine taken up was transformed into histamine. The fact that a total inhibition of histamine formation, as seen with NSD-1055 or with α HH, only reduced the uptake to about 50 per cent, indicated that some uptake could occur independently of decarboxylation. However when the incubation time was prolonged in the presence of *NSD-1055 it was found that the uptake did not proceed significantly beyond the* level reached within 10 min. One possible explanation of this finding would be that histidine transport into the cell is inhibited upon accumulation of non-decarboxylated histidine another that the cell is unable to retain histidine which is not converted into histamine and a steady-state is reached with equilibrium between the in- and outflow of histidine

Levine *et al.* (1965) who studied histidine decarboxylase prepared from rat hepatoma, found that 75 per cent inhibition was induced by NSD-1055 at a concentration of 4×10^{-7}M and by α HH at 7×10 M This is in reasonable agreement with the present results.

When the cells were exposed to the histamine liberator compound 48/80, the histamine formed *in vitro* and the endogenous histamine were released in about the same relative amounts, suggesting that they were similarly bound in the cells. How-

ever the studies on fractionated cells showed that the newly-formed histamine was recovered in the granular fraction in a significantly smaller proportion than that originally present in the cells. To explain these discrepances it may be conceived that the newly formed histamine was localized in the outer part of th granules, and thus more easily detached than the endogenous histamine when the cells were disintegrated by sonication. On the other hand, after exposure to compound 48/80 all histamine may be released by ion exchange from those granules which come into contact with the surrounding salt solution (Uvnäs and Thon 1965)

In the presence of NSD-1055 virtually none of the radioactivity taken up by the cells was recovered in the granular fraction, suggesting that histidine is not bound to the granules. This finding agrees with the statement that the decarboxylating enzyme is localized extragranularly (Hagen and Lee 1958)

The amounts of histamine formed and stored by the cells in vitro were 3—6 times higher than those previously found to be stored by mast cells incubated in histamine (Cabut and Haegermark 1966) Whereas preformed histamine seemed to be accumulated by passive diffusion into the cell and binding to the granules (Cabut and Haegermark 1966) histidine transport into the cell appeared to be mediated by a carrier mechanism. Thus the present results would confirm the concept that uptake and decarboxylation of histidine is the more important way for mast cells to build up their high intracellular stores of histamine.

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Acta physiol. scand. 1968. 73. 75—77

From the Department of Anatomy University of H lsinki, Finland

Effect of Corticosterone and 11 Desoxycortisol on the Extra Adrenal Chromaffin Tissue of the Rat

By

M. tti Lempinen and Kari Ojala

Received 6 October 1967

Abstract

Lempinen M. and K. Ojala. *Effect of corticosterone and 11-desoxycortisol on the extra-ad enal chromaffin tissue of the rat* Acta physiol. scand. 1968. *73* 75—77

Repeated injections of hydrocortisone and cortisone completely prevent the postnatal degeneration of extra-adrenal chromaffin tissue of the rat, while treatment with desoxycorticosterone (DOC) is withou such an effect. Also with massiv doses of adrenocorticotrophic hormone (ACTH) disappearance of the extra-adrenal chromaffin cells can be prevented (Lempinen 1964) In the present study the effect of corticosterone and 11-desoxycortisol on the postnatal hromaffin tissue has been studied.

Rats of the Sprague-Dawley strain were daily injected subcutaneously with 0 1 mg of cry talline corticosterone and 11-desoxycortisol in 0 1 ml of 15 % ethanol. (Ethanol showed to be the most practical solvent and only insignificant cutaneous irritation was noticed at injection sites.) Normal untreated rats and rats injected daily w th 0 1 ml of 15 ethanol served as controls. Animals were killed 10 days after birth nd the retroperitoneal tissue block containing the kidneys and the adrenals was tudied for the extra-adrenal chromaffin tissue after bichromate formol fixation (for details. see Lempinen 1964)

Animals in both experimental groups developed normally and were n good cond tion at killing, however those in corticosterone group gained more slowly in weight

Disappearance of the extra-adrenal chromaffin tissue took place in both groups The bulk of chromaffin tissue, outside the adrenals, was seen in th main para-aortic bod In the 11-desoxycortisol group, degeneration of the chromaffin cells was more marked and no difference could be noticed between them and the normal untreated controls (Fig 1 2 and 4) while in animals treated with corticosterone

The hormone preparations required for the study were kindly placed at our disposal by Messrs Organon, Oss Holland.

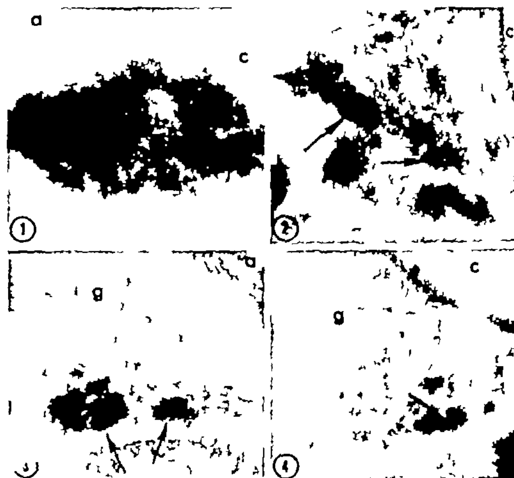


Fig. 1—4. Transverse sections through the middle part of the main para-aortic body. Bichromate formol fixation. Frozen sections 90 micra. × 200. a = aorta, c = vena cava, g = sympathetic tissue. Arrows indicate chromaffin tissue. 1. Normal newborn rat. The body gives a uniform chromaffin reaction. 2. L treated 10-day-old rat. The body is largely degenerated and almost completely non-chromaffin. Small groups of chromaffin cells are still seen. 3. Corticosterone-treated 10-day-old rat. Chromaffin cell groups can be seen in the degenerating body. 4. 11-desoxycortisol-treated 10-day-old rat. The body is almost completely non-chromaffin. Weakly chromaffin cell groups are seen in it.

chromaffin cells were regularly seen in the body. However the amount of chromaffin tissue was so small even in this group that no quantitative estimation was possible (Fig. 3). No significant differences in the intensity of the chromaffin reaction was observed between the experimental and control animals.

These results suggest that corticosterone has an effect on the extra-adrenal chromaffin tissue similar to that of cortisone and hydrocortisone, but an extremely weak one. 11-desoxycortisol, like DOC, is without any clear effect. The fact that effect of corticosterone was weak as compared with that obtained with massive ACHT injections (Lempinen 1964) suggests that ACTH affects extra-adrenal chromaffin cells

either directly or more likely through some other steroid than corticosterone which is the principal corticoid of the rat's adrenal (Bush 1953 Endróczi and Yang 1960 McCarthy *et al.* 1960, Moncola *et al.* 1959)

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Acta physiol. scand. 1968. 73. 78—92

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Effect of Directly and Indirectly Acting Sympathomimetic Amines on Adrenergic Transmitter Granules

By

U. S. von Euler and F. Lishajko

Received 9 October 1967

Abstract

Euler, U. S. v. and F. Lishajko. *Effect of directly and indirectly acting sympathomimetic amines on adrenergic transmitter granules.* Acta physiol. scand. 1968. 73. 78—92.

The actions of various sympathomimetic amines on release and reuptake of noradrenaline (NA)[1] in splenic nerve granules during incubation in phosphate buffer have been studied. The effect on the basic release rate was studied in the absence of NA in the medium. The degree of reuptake was determined with the aid of dl-³H NA. Tyramine (TA) 10^{-}—10^{-} M exerts a moderate direct releasing effect of NA from nerve granules which reaches a constant level within 10 min at 20° C. In 10^{-4}—10^{-} M concentration TA inhibits the reuptake of NA in granules. After previous loading of granules with radioactive NA, addition of TA to the incubation medium releases NA with approximately the same specific activity as that in the total store. Tritiated TA is taken up in nerve granules to a small extent approximately corresponding to the direct release of NA. The releasing action of TA is blocked by reserpine 10^{-} M, phenoxybenzamine $3 \times 10^{-}$ M and desmethylimipramine 3×10^{-4} M. Phenethylamine and amphetamine have a strong direct releasing effect and a moderate effect on reuptake of NA in granules. Ephedrine and mephentermine slightly retard NA release and inhibit NA reuptake at $3 \times 10^{-}$ M. Dopamine competitively inhibits reuptake of NA in granules. The effect of amines on release and reuptake in granules differ markedly from those exerted on the overall uptake of NA in perfused organs.

In 1931 Burn and Tainter discovered that the denervated iris was insensitive to tyramine (TA). This and other observations led Fleckenstein (1953) and Burn and Rand (1958) to postulate that TA might act by liberating noradrenaline (NA) from nerve endings, a concept which later has become generally accepted and supported by various findings. Thus Carlsson *et al.* (1957) observed that TA was without action in an animal whose NA stores had been depleted by reserpine and Burn and Rand (1958) were able to temporarily restore the effect of TA by infusion of NA in the reserpine-depleted animal.

A direct proof of the releasing effect of TA on the NA stores was given by Stjärne (1961) in experiments on the perfused spleen. A releasing effect on isolated storage

[1] Abbreviations used: NA (noradrenaline), TA (tyramine), DA (dopamine), ATP (adenosine triphosphate), PBA (phenoxybenzamine), MAO (monoamine oxidase), DMI (desmethylimipramine).

granules from the adrenal medulla and from adrenergic nerves was shown by Schümann (1960) and by Euler and Lishajko (1960). The latter authors also found that TA in concentration of 10^{-3} M was without action. Schümann and Philippu (1961) concluded from their experiments that TA substituted stoichiometrically NA in the granules and found that TA had no releasing action at low temperature.

An inhibitory action of TA on the uptake of radioactive NA in tissues was observed by Hertting, Axelrod and Whitby (1961) on the rat heart in vivo, by Dengler, Spiegel and Titus (1961) on isolated tissue slices, by Burgen and Iversen (1965) on the isolated perfused rat heart and by Muscholl (1960) on the rat heart. These findings led Stjärne (1966) to postulate that the releasing effect of TA on the NA stores might be due to inhibition of reuptake in the tissues.

Trendelenburg (1967) observed that the action of TA on the isolated heart could be restored even if the organ content of NA was very small and concluded *that TA released NA from a small "pool"*.

By using high doses of TA several authors have observed a depletion of the NA content of various organs (Bhagat 1964, Gutman and Weil-Malherbe 1966) and it has also been found that TA causes tachyphylaxis.

While some evidence thus speaks in favour of the concept that TA exerts a direct releasing effect on the NA stores in the axon terminals, the inhibitory effect on reuptake is an alternative which must be considered. In the present paper we wish to report the results of studies of the effect of TA and other indirectly amines on the release and uptake of NA in isolated adrenergic nerve granules. In addition some results with other sympathomimetic amines will be reported.

Methods

Nerve granules were prepared from bovine splenic nerves obtained from the slaughter house and transported on ice to the laboratory. After preparation and dessheathing, the nerves were cut in small pieces and homogenized in an Ultra Turrax apparatus (Janke & Kunkel, Freiburg) in 0.13 M potassium phosphate pH 7.5 at ice water temperature. For 1 g of nerve tissue about 10 ml buffer was used. After removal of coarse particles by centrifugation in 10 ml plastic tubes at $9000 \times g$ for 10 min, the supernatant, containing most of the NA granules was used for incubation experiments. The endogenous NA in the supernatant under these conditions is $1-3 \times 10^{-6}$ M.

After incubation of the granule suspension, usually at 20° for 60 min, the granules were sedimented by centrifugation 30 min at $50,000 \times g$ in a refrigerated centrifuge. The sediment was extracted with 0.4 M potassium perchlorate, and NA measured fluorimetrically. No correction was made for the recovery of 70—80 per cent. NA in the supernatant was adsorbed on alumina and eluted by 0.25 N acetic acid and determined fluorimetrically.

The following standard experiments were made:

1. Determination of the NA release rate at various concentrations of NA and other amines in the medium with and without addition of adenosine triphosphate (ATP) in concentrations of 1—3 mM, added at 0 and 30 min.
2. Determination of net uptake of NA during 30 min at 20° after partial depletion of the NA in the granules by preincubation for 10 min at 37°.

Basic release rate was determined by incubation in the presence of 5×10^{-4} M potassium ferrocyanide (Euler and Lishajko 1967). Release rate was expressed by the release constant $k = \frac{2.3}{t} \log \frac{100}{p}$, where p stands for the remaining NA after incubation in per cent of the preincubation value. Half-time $(t/2) = \frac{0.693}{k}$.

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[1] Abbreviations used NA (noradrenaline) TA (tyramine) DA (dopamine) ATP adenosine triphosphate) PBA (phenoxybenzamine) MAO (monoamine oxidase) DMI (desmethylimipramine)

It can also be noted that at a TA concentration of 3×10^{-4} M the release is largely independent of the NA concentration in the medium, indicating effective inhibition of reuptake.

At TA concentrations of 10^{-4}—3×10^{-4} another phenomenon can be observed in that the release rate tends to decrease. At still higher TA concentrations, 10^{-3} M, the fall in release rate is obvious and since this can be seen also in the presence of Fe^{3+} when no reuptake occurs it must be ascribed to a diminished release rate.

The shape in the curves in Fig. 1 obtained with different concentrations of NA in the medium also reveals that when the NA level is high, 10^{-5} or 10^{-4} M, TA is becoming increasingly less active in inhibiting reuptake. While TA 10^{-5} M is quite effective when NA is present in 1—3×10^{-6} M it has only little effect at NA 10^{-5} M and virtually no effect at NA 10^{-4} M. Apparently TA can only exert an inhibitory effect on reuptake of NA into the granules as long as it is present in a competitive concentration.

B. Incorporation of dl H NA at various NA and TA concentrations with and without ATP Mg^{2+}

The incorporation of radioactive NA in the granules during incubation for 60 min at 20° can be seen in Fig. 2. In these series the NA concentration was 1—$3\times$ ^{6} M. From the figure it is seen that from 3×10^{6} M TA on, there is a gradual decrease in the proportion of incorporated labelled NA, so that at a TA concentration of 10^{-4} M there is only a small reuptake. Since at this concentration there is still no *inhibition of the basic release, the total releasing effect is maximal.* At 3×10^{-4} M TA inhibition of reuptake is less complete but there is on the other hand some direct releasing effect (Fig. 1). The effect of TA on the relative reuptake of NA at concentrations of 1—3×10^{-6} M in the medium is seen in Fig. 3.

At higher NA concentrations the inhibitory effect of TA on the reuptake depends on the relative concentrations of the two amines. In general the concentration of TA has to be some 10 times higher than that of NA in order to exert a clearcut

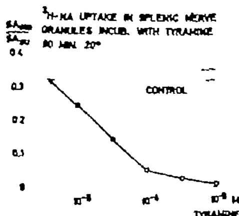


Fig. 2. Splenic nerve granules incubated in phosphate buffer 60 min at 20°. NA conc. in medium 1—$3\cdot10^{-6}$ M. Ordinate: Proportion of NA incorporated by reuptake. Abscissa: TA conc. in medium.

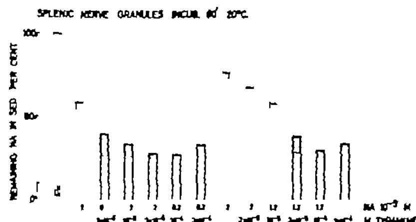


Fig. 3. Splenic nerve granules incubated in phosphate buffer 60 min at 20° with NA and TA as indicated. Ordinate: Per cent remaining NA in granules after incubation. Stippled part of column labelled NA, taken up from the medium.

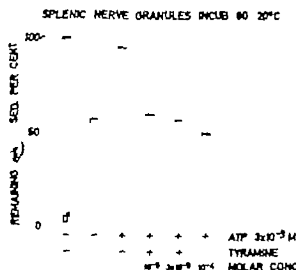


Fig. 4. Splenic nerve granules incubated in phosphate buffer 60 min at 20°. NA conc. in medium 1.5×10^{-6} M. Ordinate: Per cent remaining NA in granules after incubation. Stippled part of column labelled NA.

inhibitory effect (Fig. 5). At a NA concentration of 10^{-5} M, TA 5×10^{-5} M thus has a markedly smaller inhibitory effect on the reuptake than at NA 10^{-6} M.

If reuptake is stimulated by addition of ATP Mg^{2+} to the incubation medium this effect is also inhibited by TA as seen in Fig. 4. Almost all of the extra reuptake caused by ATP at a NA concentration of 1.5×10^{-6} M is annulled by TA 10^{-4} M.

The net uptake effected by ATP in the presence of NA 10^{-6} M after previous partial depletion is largely inhibited by TA 10^{-5} M (Fig. 5). If NA is present in 10^{-5} M concentration, the inhibitory effect of TA 10^{-5} M is only slight, however and only at the TA concentration 10^{-4} M the extra reuptake is abolished.

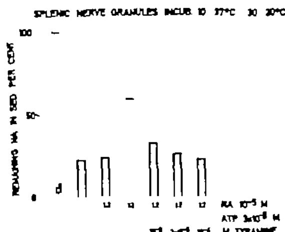


Fig. 5. Splenic nerve granules pre-incubated 10 min at 37° and re-incubated 30 min at 20° with NA, TA and ATP as indicated. Stippled part of column labelled NA.

C. *Release of ³H NA from granules by TA*

In order to establish whether TA releases NA uniformly from the granules, experiments were made in which the granules were allowed to take up labelled NA before addition of TA to the medium.

Granules were incubated for 30 min at 20° in the presence of 10^{-4} M NA containing a tracer dose of dl-³H NA. After incubation the granules were twice sedimented and resuspended in fresh buffer and incubated for 60 min at 20° together with TA in concentrations from 10^{-4} M to 3×10^{-4} M. After incubation and sedimentation, radioactivity and specific activity were measured in the sediment and in the supernatant and also in the sediment before the second incubation.

In Table I are given the relative values of the specific activities of the sediment and the percentage remaining NA after incubation.

The data in Table I indicate that if there is any difference in the specific activity at all between the NA spontaneously released and under the influence of TA

TABLE I. Isolated bovine splenic nerve granules preincubated with dl-³H NA. Relative specific activities and amounts of remaining NA after second incubation with TA. Spec. act. of sediment before second incubation = 1. Mean of 2 expts.

TA added (molar conc.)	—	10^{-4}	3×10^{-4}	10^{-4}	3×10^{-4}	10^{-4}	3×10^{-4}
Relative spec. act. of NA in sed. after incubation	1.00	1.09	1.11	1.12	1.10	1.13	1.08
Per cent remaining NA in sed. after incubation	31	40	3[illegible]	33	32	30	3[illegible]

TABLE II. Splenic nerve granules incubated at 20°. Uptake of TA from the medium containing H TA 10^{-4} M and release of NA in the presence of ferricyanide 5×10^{-4} M

Incubation time, min	10	20	30	45	60
Uptake of TA, µg per tube	0.045	0.047	0.055	0.067	0.063
Release of NA, µg per tube	0.05	0.07	0.08	0.08	0.06

this is at most quite small. It can therefore be concluded that TA releases NA chiefly from the same granular population or from the same granular sites as in spontaneous release.

D. *Uptake of radioactive TA*

In a series of experiments radioactive TA was added to the incubation medium and the uptake of radioactivity measured. The corresponding amount of TA taken up was compared with the amount of NA released in excess of the control. To the incubation medium was also added Pargyline in a concentration of 5×10^{-5} M which inhibits monoamine oxidase (MAO) but does not affect the release rate of NA from the granules. By addition of ferricyanide the error due to inhibition of reuptake of NA could be avoided.

As seen in Table II the amount of TA taken up corresponded fairly well with the amount of NA released in the presence of ferricyanide. The radioactivity found in the sediment after incubation is almost entirely due to octopamine formed from TA during the incubation.

While the absolute amounts of TA equivalents in the granules are approximately the same after 10 and 60 min of incubation the amounts in proportion to the NA remaining in the granules reaches a plateau after 30 min incubation, presumably due to continuous release of the octopamine formed.

The results confirm the observations of Schümann and Philippu (1961) of a stoichiometric exchange between TA and NA in the granules.

After uptake of radioactive TA during incubation for 30 min at 20° in the presence of ATP and 2 × 10 M TA the release of NA and of TA equivalents, measured as radioactivity was followed during a second incubation for 30 and 60 min at 20°. The amounts of TA eq and NA remaining in the granules after incubation were roughly proportional. The TA eq values are calculated from the radioactivity in the sediment but represent mainly octopamine in the granules.

E. *Interaction of tyramine and reserpine phenoxybenzamine (PBA) and desmethylimipramine (DMI)*

Reserpine

It has previously been shown (Euler and Lishajko 1961 a) that the NA releasing

TABLE III. Bovine splenic nerve granules incubated 60 min 20° in phosphate buffer. To each tube added ³H TA in a concentration of 0.9×10^{-4} M. Non-labelled TA and reserpine 10^{-4} M added as indicated

Tube no.	Added TA M (total)	µg NA per tube in sed. after incub.		Uptake of TA in µg per tube.	
		—	Res. 10^{-4} M	—	Res. 10^{-4} M
1	0.9×10^{-4}	3.0	—	[illegible]*	—
2, 3	0.9×10^{-4}	1.7	2.8	0.10	0.005
4	4×10^{-4}	1.3	—	0.14	—
5, 6	10^{-4}	1.1	2.8	0.17	0.049
7, 8	3×10^{-3}	1.0	2.9	0.20	0.069
9, 10	10^{-4}	1.1	2.8	0.13	0.16

* not incubated

effect of TA 7.3×10^{-5} M on nerve granules is abolished by reserpine in a concentration of 10^{-4} M.

From Table III it can be seen that reserpine 10^{-4} M strongly inhibits the uptake of radioactive TA. At a concentration of 0.9×10^{-4} M in the medium (tube no. 3) the uptake of TA is only 5 per cent of that taken up without reserpine, indicating that prevention of TA effects by reserpine may take place at the granular level and depend on lack of uptake of TA in the granules. This is further illustrated in tubes no. 5 and 6 in which the NA releasing effect of TA is abolished by reserpine. The implication is that reserpine can inhibit or prevent TA action under certain conditions even if the granular stores are not depleted.

Phenoxybenzamine (PBA)

Like reserpine, PBA retards the spontaneous release of NA from nerve granules (Euler and Lishajko 1961 b, Euler, Stjärne and Lishajko 1964).

As seen in Table IV, PBA in a concentration of 3×10^{-4} M almost completely prevents the NA releasing action of TA 10^{-4} M and in this respect is reserpine-like.

TABLE IV. Bovine splenic nerve granules incubated 60 min 20° in phosphate buffer pH 7.5. NA concentration in medium 1.2—3.1 10^{-4} M (3 expts)

Tube no.	Added		NA in sediment after incub. in per cent of original amount (mean and range)
	TA	PBA	
1 (control)	—	—	53 (46—57)
2	—	$3 \cdot 10^{-4}$ M	80 (78—83)
3	10^{-4} M	—	28 (22—32)
4	10^{-4} M	$3 \cdot 10^{-4}$ M	77 (74—78)

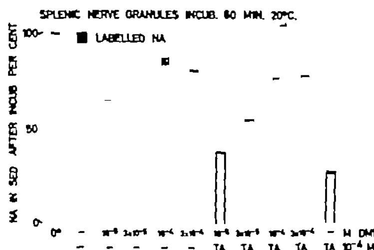


Fig. 6. Splenic nerve granules incubated in phosphate buffer 60 min at 20° with DMI and TA as indicated. NA conc. in medium $1-3 \times 10^{-6}$ M. Ordinate: Per cent remaining NA in granules after incubation. Stippled part of column: labelled NA.

Desmethylimipramine (DMI)

DMI is particularly known for its ability to block the reuptake and uptake of NA at the axon membrane. When tested on the release of NA from nerve granules it can be seen (Fig. 6) that it has a considerable inhibitory effect which is combined with a marked inhibition of the incorporation of labelled NA. Thus at a concentration of 3×10^{-4} M DMI almost completely blocks reuptake of NA in the granules.

The NA releasing effect of TA 10^{-4} M (chiefly by inhibition of reuptake) is abolished by DMI in concentrations of 3×10^{-5} M and stronger.

II. *Other sympathomimetic amines*

A. *Effects on basic NA release rate*

The effect of a number of sympathomimetic amines on the basic release rate of NA from nerve granules has been tested in the presence of ferricyanide.

Table V shows the effect of these amines in a concentration of 10^{-4} M on the basic release rate and in the presence of NA $1-3 \times 10^{-6}$ M.

From Table V it can be seen that the following amines in a concentration of 10^{-4} M have a direct NA releasing effect on isolated nerve granules in decreasing order: Phenethylamine, phenylethanolamine, methamphetamine, amphetamine, TA, while the remainder has no such effect. On the other hand the first row shows that in addition to those enumerated above octopamine, propadrine and metaraminol have a net releasing effect in the presence of NA $1-3 \times 10^{-6}$ M. Of these octopamine and propadrine have no effect on the direct release rate and it may therefore be concluded that they inhibit reuptake. This is also borne out by experiments in which the uptake of radioactive NA was estimated (see below), although the inhibitory

TABLE V. Bovine splenic nerve granules incubated 60 min at 20° in phosphate-buffer pH 7.5 in the presence of NA $1-3\times10^{-4}$ M and without NA. Amines added to 10^{-4} M. Remaining NA in granules after incubation. Mean of 3—6 expts. Relative reuptake of NA expressed as SA_AD/SA_AC

Amines added 10^{-4} M	Per cent remaining NA in sediment. Release constant $\times10^{-3}$ within parenthesis: NA $1-3\times10^{-4}$ M	without NA	SA_AD / SA_AC
NA $1-3\times10^{-4}$ M	53 (1.1)	—	0.30
Without NA	—	34 (1.8)	—
Phenylethylamine	22 (2.5)	16 (3.1)	0.025
Phenylethanolamine	24 (2.4)	22 (2.5)	0.11
Methamphetamine	28 (2.1)	24 (2.4)	0.086
Amphetamine	4 (2.4)	26 (2.5)	0.084
Tyramine	28 (2.1)	31 (2.0)	0.05
Propadrine	36 (1.8)	3[illegible] (1.7)	0.15
Octopamine	35 (1.7)	37 (1.7)	0.056
Ephedrine	56 (0.96)	49 (1.2)	0.20
Metaraminol	43 (1.3)	49 (1.2)	0.055
Mephentermine	60 (0.84)	53 (1.1)	0.[illegible]7
Dopamine	(48) (1.2)	—	0.023

effect of propadrine is fairly weak. A retardation of release is observed for metaraminol.

Ephedrine and mephentermine belong to a group of amines which retard the NA release from granules and have only little action on the reuptake of NA in the concentration used.

Dopamine (DA) causes no effect on the net release of NA from granules either at high or low NA concentrations. However, since it strongly inhibits reuptake of NA into the granules (see below) the effect is in all likelihood due to β-hydroxylation of DA to NA in the granules which tends to mask the net loss.

The same amines as listed above have also been tested for their effect on granules in the concentration 10^{-5} M. The results were similar to those obtained at 10^{-4} M as regards the direct release effect. The inhibitory action on reuptake of NA in the presence of NA 10^{-4} M was, however, smaller when the amines were present in concentrations of 10^{-5} M, particularly for phenethylamine and phenylethanolamine.

B. *Effect of sympathomimetic amines on reuptake of NA*

By estimation of the incorporation of radioactive NA into the granules in the presence of $1-3\times10^{-4}$ M NA during incubation for 60 min at 20° the effect of various sympathomimetic amines in different concentrations on the reuptake of NA has been studied. The results are presented in Fig. 7.

From the figure it can be seen that the inhibitory effect of amines on reuptake

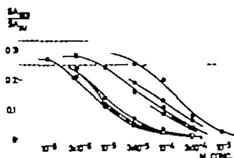


Fig 7 Splenic nerve granules incubated in phosphate buffer 60 min at 20° with sympathomimetic amines in different concentrations. NA conc. in medium 1—3×10⁻⁶ M. Ordinate Proportion of NA incorporated by reuptake

varies considerably It is greatest for DA, phenethylamine and metaraminol and is weakest, on a molar basis, for ephedrine. Mephentermine 10^{-4} M in a single determination, had the same weak effect as ephedrine, while octopamine and amphetamine 10^{-} M were in the same range as metaraminol and methamphetamine respectively

The values for the relative reuptake of ^{3}H NA during incubation 60 min at 20° in the presence of the amines tested are given in Table V There is obviously no correlation between the degree of inhibition of reuptake and the direct releasing effect. Thus metaraminol strongly inhibits uptake in the same way as phenethylamine but retards the release while phenethylamine accelerates it. DA is also a strong inhibitor of reuptake of NA (see below)

The effect of the sympathomimetic amines on the net uptake of NA after partial ...tion of the granules by preincubation at 37 for 10 min was of a similar kind ... that described above for reuptake. This also holds true for adrenaline which in a concentration of 10^{-} M has a moderate inhibitory effect on reuptake of NA 10^{-6} M

C. *Interact on of DA w th lease and uptake of NA in granules*

As shown in Fig 7 DA exerts a strong inhibitory influence on the reuptake of NA into granules at various concentrations of NA in the medium, similar to that of metaraminol and phenethylamine (Table VI)

From the table it can be seen that DA over the range 10^{-6} M to 10^{-} M exerts no apparent action on the NA release rate. On the other hand the incorporation of radioactive NA falls rapidly with increasing concentration of DA. At DA 10^{-4} M the reuptake of NA is less than 10 per cent of the original. The constancy of the NA values in the sediment must therefore be due to either a gradually diminishing direct release rate or transformation of DA to NA in the granules. Since there is no indication of an inhibition of the release rate (tested at low NA in the medium) the NA values in the sediment indicate an increasing formation of NA from DA which becomes marked from a DA concentration of 10 M on.

When DA and NA are both present in a concentration of 10^{-6} M the reuptake of NA is inhibited by about 40 per cent. The unaltered NA content in the granules

TABLE VI. Bovine splenic nerve granules incubated 60 min at 20° in phosphate buffer dl-H-NA added in conc. 2×10^{-4} M. NA and DA concentrations as indicated. Remaining NA in sediment after incubation in per cent of original amount. Relative uptake of NA given as SA_{sed}/SA_{sc}

DA M conc.	NA M conc.	Remaining NA in sediment (per cent)	$\frac{SA_{sed}}{SA_{sc}}$
—	1.4×10^{-4}	51	0.28
10^{-4}	1.4×10^{-4}	52	0.27
3×10^{-4}	1.4×10^{-4}	50	0.21
10^{-4}	1.4×10^{-4}	50	0.11
3×10^{-4}	1.4×10^{-4}	50	0.05
10^{-4}	1.4×10^{-4}	49	0.023
—	—	32	—
—	$1.14 + 10^{-4}$	68	0.39
10^{-4}	1.14×10^{-4}	62	0.29
10^{-4}	1.14×10^{-4}	63	0.22

indicates new formation of NA. After previous partial depletion, DA in a concentration of 10^{-4} M also strongly inhibits the net uptake of NA.

D *Uptake of H DA in granules*

The uptake of tritiated DA in granules was measured after incubation with DA in concentrations of 10^{-4} M to 10^{-4} M. The results are given in Table VII.

The amounts of DA estimated by the radioactive uptake in the sediment correspond reasonably well with the excess NA found in the granules if inhibition

TABLE VII. Bovine splenic nerve granules incubated 60 min at 20° in phosphate buffer. H-DA added in conc. 10^{-4} M. NA and DA conc. in medium as indicated

DA M conc.	NA M conc.	NA in sed. µg	DA uptake in sed. µg	Reuptake µg NA calc. found	µg NA found	Excess NA in sed. µg
—	—	0.45	—	—	—	—
—	2.1×10^{-4}	0.77	—	0.32	0.32	0
10^{-4}	2.1×10^{-4}	0.78	0.032	0.31	0.34	0.03
3×10^{-4}	2.1×10^{-4}	0.77	0.068	0.24	0.32	0.08
10^{-4}	2.1×10^{-4}	0.77	0.15	0.13	0.32	0.19
3×10^{-4}	2.1×10^{-4}	0.77	0.25	0.057	0.32	0 ?6
10^{-4}	2.1×10^{-4}	0.81	0.37	0.026	0.36	0.33
—	1.2×10^{-4}	1.1	—	0.65	0.63	0
10^{-4}	1.2×10^{-4}	1.0	0.018	0.48	0.55	0.07
10^{-4}	1.2×10^{-4}	1.0	0.12	0.37	0.53	0.18

Calculated from the values of SA_{sed}/SA_{sc} in Table VI

of reuptake of NA is taken into account. This suggests that the DA taken up has been transformed to NA. In the presence of NA 1.2×10^{-5} M the excess NA uptake is somewhat larger owing to increased reuptake of NA.

The net uptake of DA after partial depletion yielded similar results in principle the uptake agreeing reasonably well with the excess NA in the granules when allowance is made for the inhibition of NA uptake caused by DA.

Discussion

An analysis of the action of various sympathetic amines among them some which are generally characterized as indirectly acting has shown a dual mode of action at the granular level Thus the present work supports both concepts as to the action of these amines direct release by stoichiometric substitution (Schümann and Philippu 1961 and inhibition of reuptake of transmitter into the tissues. In the latter case it has not been explicitly stated whether the action is considered to be at the axon membrane or at the granular level (cf Burgen and Iversen 1965, Stjärne 196[illegible]

In order t differentiate between the two kinds of action on the transmitter granules it has become necessary to introduce two procedures. The basic release rate can only be studied in the absence of NA in the medium which has been achieved by addition of ferricyanide to the incubation medium. The second process which had t be elucidated in detail was the reuptake into the granules at different NA level which as studied with the aid of labelled NA (Euler and Lishajko 1967 b In this wa it became possible to determine whether addition of an amine accelerated or retarded the NA release in the absence of reuptake and also to complement these results by measuring the action on reuptake of NA at a given NA level In most ases this has been the concentration in the medium obtained during the standard p eparation of the granule suspension. This concentration is $1-3 \times 10^{-6}$ M NA and gi es by reuptake an apparent release rate which is about half that of the basic ele se at and convenient for estimation of inhibition of reuptake of NA

Acceleratio f NA reuptake does not seem to occur with the amines tested and has so f l been observed with various nucleotides, particularly ATP which is conside bl more activ than CTP ITP and UTP which have about half the action of ATP n onc nt tion of 3 mM in the presence of Mg

The uptake of TA and DA n the granules was studied with the aid of tritiated amines. Both amines are tak n up to some extent, especially when present in higher concentrations n the medi 10 M and higher) Both amines are transformed to the corresponding β-hyd oxylated amines. While the octopamine produced by TA by itself has no direct lea ng effect t inhibits reuptake of NA. DA on the other hand is transformed t NA which remains in the granules. Although DA inhibits reuptake of NA it seem d at t has no net releasing effect on the granules owing to new formation of NA. Whether t exerts a net release of NA *in vivo* can not be decided by these experiments but may well be the case E idence for such release

has, in fact, been given by Potter and Axelrod (1963) and by Harrison, Levitt and Udenfriend (1963).

All amines with a net releasing action should be able potentially to deplete the stores. This has been demonstrated for a variety of organs with TA (Gutman and Weil-Malherbe 1966) as well as for phenethylamine (Jonsson, Grobecker and Holtz 1966) and for several others.

Some of the studied amines exerted only a negligible action on the reuptake like ephedrine and mephentermine, at least in the concentrations 10^{-5}—10^{-4} M, in which several other amines were strongly active. In a concentration of 3×10^{-4} M ephedrine inhibited reuptake of NA requiring, however, about 30 times higher concentration than e.g. metaraminol or phenethylamine.

It is of interest to compare the results obtained on nerve granules with the overall effect on uptake studied on the isolated rat heart by Burgen and Iversen (1965). In their experiments the NA concentration was lower, however, (0.6×10^{-7} M) than in our experiments on uptake in granules (1—3×10^{-6} M). The relative affinities for uptake calculated in the same way as in the experiments of Burgen and Iversen showed large differences. While TA, amphetamine and methamphetamine showed very nearly the same affinity values in our study on granules, their affinity values for tissue uptake in Burgen and Iversen's experiments were 245, 610 and 165. Metaraminol had in our study the same affinity value as phenethylamine but had more than 14 times higher value than phenethylamine in Burgen and Iversen's experiments. These differences suggest that the uptake by the axon membrane constitutes an important factor in the overall uptake. In no case was the affinity value in Burgen and Iversen's study higher than in ours if the value for phenethylamine was set as 100. Only for mephentermine, (+)amphetamine and metaraminol did the ratio between the affinity values in the two studies exceed a factor of 10.

By comparing the results obtained in the present study and those on the overall uptake it seems possible to gain some information about the role of the axon membrane in the uptake process. Obviously there exist important differences in the uptake processes at the axon membrane and the nerve granules.

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The Secretion of Sodium and Potassium in Cat Submandibular Saliva during the First Period after Start of Stimulation

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Abstract

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In a previous publication the existence of a positive sodium rest transient in the cat submandibular saliva was shown. This sodium transient was greater and had a longer duration than the well known positive potassium rest transient. In the present work a negative potassium transient succeeding the positive potassium transient and in many respects being closely linked to the positive sodium transient, has been demonstrated. This negative potassium transient, as well as the positive potassium transient, is also reflected in the venous outflow from the gland.

In a previous publication (Petersen and Poulsen 1967) a positive sodium rest transient in the cat submandibular saliva was described. The existence of a positive sodium transient means that the sodium concentration in the first period after start of secretion is increased compared with steady state conditions. The sodium transient was quantitatively expressed as the transient excess in sodium concentration, which was defined as the difference in concentration between a transient and a steady state sample of saliva taken at the same secretory rate.

When a previously resting gland is stimulated and starts secreting, the gland tissue loses potassium to the saliva as well as to the blood. This is known as the positive potassium rest transient and is demonstrated by the temporarily increased potassium concentration in the saliva and in the venous outflow from the gland (Burgen 1956). Potassium transients have also been demonstrated after shifts in stimulation frequency (Burgen and Emmelin 1961).

In the acini a primary secretion is produced with a composition close to an ultrafiltrate of plasma with respect to the concentrations of sodium, potassium and chloride (Young and Schögel 1966 and Martinez, Holzgreve and Frick 1966).

the duct system the main processes are a reabsorption of sodium chloride in excess of water and a secretion of potassium which probably take place in the striated ducts (Young and Schögel 1966 Thaysen 1960 and Petersen and Poulsen 1967)

The purpose of the present work was to test the hypothesis presented by Petersen and Poulsen (1967) that the sodium transient is due to a smaller reabsorption of sodium during the transient period than during the steady state period. As it seems likely that the reabsorption of sodium in the duct system is linked to the secretion of potassium, it would be a good argument in favour of the hypothesis if the positive sodium transient was accompanied by a negative potassium transient. It was found that the positive potassium transient is followed by a negative potassium transient and that the sodium and potassium concentration reach the steady state level at the same time

Methods

Cats weighing 2–3 kg were anesthetized with chloralose (70—90 mg/kg i.p.) All details about the preparation of the chorda lingual nerve and the duct about stimulation of the gland, collection of the submandibular saliva and the measurement of sodium and potassium concentrations in the saliva have been described in a previous publication (Petersen and Poulsen 1967

In some experiments also the concentration of sodium and potassium in the venous outflow from the submandibular gland was measured. In these cases all tributaries to the external jugular vein were ligated except those draining the submandibular gland. Thereafter 5—8 ml 300 mg% heparin was given and the external jugular vein was cannulated with polyethylene tubing. The venous samples were centrifugated immediately after they had been collected and the plasma was drawn off. Sodium and potassium concentrations were determined on the plasma samples.

The first step in an experiment was to obtain the steady state relationship between the sodium and potassium concentration in the saliva and the secretory rate. The criterion for steady state condition was the same as previously (Petersen and Poulsen 1967) Thereafter the gland was allowed to rest for 1 hr and a transient curve was obtained. Throughout the transient period which normally lasted 15 min a constant stimulation frequency was maintained. In Fig. 1 is seen the steady state curves and the transient values for sodium from one transient period from one experiment. The transient excess sodium concentration, at the time where a transient sample has been obtained, is the vertical distance between the transient value and the steady state curve. In Fig. 2 the transient excess sodium concentration determined this way from Fig. 1 is shown as a function of the time passed after start of the stimulation

The reproducibility of the linear part of the steady state curve in the same gland was tested in 8 glands. The result is shown in Table I. The time passed between the two experi-

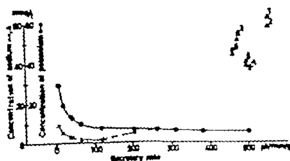


Fig. 1. Saliva sodium and potassium concentration as a function of the secretory rate – – – × = steady state sodium concentration. ———○ = steady state potassium concentration. △ = sodium concentration during the transient period. (The figures indicate the consecutive order in which the values were obtained. Because of lack of space only some of the figures are shown.) The vertical distance between the transient values and the sodium steady state curve represents the transient excess sodium concentration.

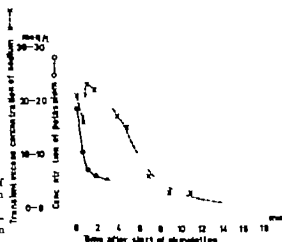


Fig. 2. Transient excess concentration of sodium (×– – –×) and concentration of potassium (○———○) in the saliva as function of time after start of stimulation. The sodium values are derived from Fig. 1. Stimulation frequency 10 cps.

Table I. Reproducibility of the linear part of the sodium steady state curve in the same experiment

Expt. no.	28a	28d	29	30	31	34	35a	35d
1. period								
slope (meq/l/µl/min/g)	0.19	0.13	0.14	0.23	0.40	0.13	0.12	0.40
y-intercept (meq/l)	−3	+1	+6	+13	+13	+1	0	0
2. period								
slope (meq/l/µl/min/g)	0.10	0.13	0.12	0.25	0.33	0.17	0.12	0.36
y-intercept (meq/l)	+3	+3	+9	+13	+13	+1	−1	+10

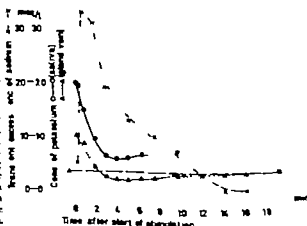


Fig. 3. Transient excess concentration of sodium in the saliva (×– – –×) concentration of potassium (○———○) in the saliva and concentration of potassium in the venous outflow from the gland (△– – –△) as function of time after start of stimulation. Additionally the potassium concentration in the gland vein during the resting period (▲) and the potassium concentration in plasma obtained from the femoral vein after the transient period (■) are shown. Stimulation frequency 10 cps.

mental period was 1—3 hrs. The steady stat sodium curv shown in Fig. 1 is variant upon the standard sodium curv shown in Fig. 3 in our previous paper (Petersen and Poulsen 1967). In the present work this variant was obtained in 4 out of 28 expts. In 21 expts. the standa d sodium curve (expts. no 28 a, 28 d, 29 30 34 and 35 in Table 1) was obtained and in the emaining 3 expts. somewhat steeper curv (expts. no 31 and 33 d in Table 1) is seen.

When working w th frequency shift transients, the first 3 drops of saliva (corresponding to about 60 l roughly the ol me of the duct system+the duct cannula) were discarded.

Results

Negat ve potassium and positive sodium rest trans ents

An examination of the potassium concentration in the saliva and the venous blood from the gland revealed, that the positive potassium transient was followed by a negative potassium transient. The steady state level for the potassium concentration in both saliva and in the venous blood from the gland was reached at the same time as the sodium concentration in the saliva reached the steady state level. In Fig is hown the result from one experiment demonstrating a positive sodium transient in the saliva and both positive and negative potassium transients in both the saliva and the venous blood from the gland. Three experiments with simultane ous measu ement of sodium and potassium in the saliva as well as the venous blood from th gland were carried out. Two of these experiments had the course shown in Fig. 3. In the third experiment only a very delayed and small sodium transient was present. I this ase also small and delayed negative potassium transients were seen Fig. 4.

In l out of 25 expts. where sodium and potassium concentrations were only determ ned n the sali a, sodium and potassium concentrations reached steady state evel t the same time

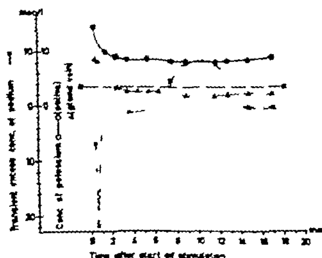


Fig. 4. The same as Fig. 3.

Table II. Reproducibility of positive sodium and negativ potassium transient in the same experiment

Expt. no.	127	128	133	147
1. period				
Max. size of positive sodium transient (meq/l)	23	32	24	22
Max. size of negative potassium transient (meq/l)	0.4	0.4	0.8	1.2
2. period				
Max. size of positiv sodium transient (meq/l)	22	27	18	26
Max. size of negativ potassium transient (meq/l)	0.6	0.4	1.0	0.8

The negative potassium transients in the saliva cannot be an artefact due to changes in the secretory rate during the transient period, as the secretory rate in the transient periods of all experiments was so high that the potassium concentration was independent of the secretory rate (Petersen and Poulsen 1967). The possibility that the negative potassium transient in the saliva should be an artefact due to interference between sodium and potassium during the measurement in the flame photometer was investigated. During the period of the sodium transient, sodium concentration often changes very considerably in some cases from 100 meq/l to about 30 meq/l. In a control experiment, however the potassium concentration was uninfluenced by such great changes in th sodium concentration.

The maximal transient excess in sodium concentration in 16 expts., in which transients were evoked with a stimulation frequency of 10 cps after a previous resting period of 1 hr varied between +53 and −1 meq/l with a mean value of 19 meq/l±4 (S.E.) The maximal size of the negative potassium transient (i.e. the difference between the minimal potassium concentration during the transient period and the steady state concentration) varied in the same 16 expts. between —2.2 and +0.2 meq/l with a mean valu of —1.0±0.2 (S.E.)

In 4 expts the transients were recorded twice with a time difference of 4—10 hrs. The result is shown in Table II.

Sodium transient immediately after a shift in stimulation frequency

When the stimulation frequency was increased suddenly a positive transient was seen very often In Fig. 5 an example of such a transient is shown. Negative transients were never seen when the stimulation frequency was increased When the stimulation frequency was suddenly decreased, sometimes negative transients sometimes positive transients were seen. In Fig. 6 examples all taken from the same gland are shown The sizes of the frequency shift transients were very variable from 1—2 meq/l to 40 meq/l. The duration of the transients was always smaller than the duration of the rest transients. Normally the change in stimulation frequency associated with a corresponding change in secretory rate, but the change in ...

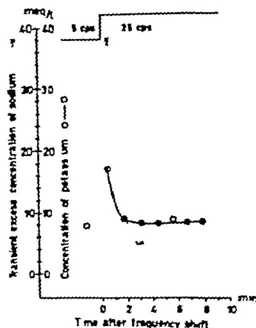


Fig 5 Transient excess concentration of sodium (— — —) and concentration of potassium (———) in the saliva as function of time after frequency shift.

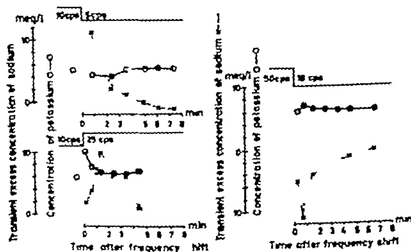


Fig 6 The same as Fig

rate was not a necessary factor for the presence of a transient. In the experiment shown in Fig 5 no change in the secretory rate was noticed when the stimulation frequency was changed

Discussion

After the first 2 min of a t l or period the saliva in the transient period has a high sodium and a low potas u oncentration compared with stead tate conditions. The sodium concentration however never exceeds the acinar fluid sodium

concentration and it seems that the potassium concentration never decreases significantly below the acinar fluid potassium concentration. From this it appears natural to postulate, that the positive sodium and negative potassium transients are due either to a smaller sodium reabsorption and potassium secretion during the transient period than during the steady state period, or that the sodium and potassium permeability in a part of the duct system situated orally to the sodium reabsorbing part is increased in the transient period compared with the steady state period. The latter explanation would require that there were electrochemical gradients in that part of the duct system favouring influx of sodium and outflux of potassium. For sodium this is the case (Petersen and Poulsen 1967) With respect to potassium it is more uncertain. The potassium concentration is higher in the duct fluid than in the interstitial fluid because of the potassium secretion in the striated ducts, but the electrical gradient opposes potassium outflux. There is no direct measurement of the transductal potential difference (P.D.) in the region of the striated ducts in salivary glands. In some functionally analogous duct systems P.D.s from —50 to —90 mV (lumen negative) have been measured The Whartonian duct of the rat submandibular gland (Young *et al.* 1967) the duct of the human sweat gland (Schulz *et al.* 1965) and the distal rat kidney tubule (Frömter and Hegel 1966) It seems reasonabl to assume that a transductal P.D of the same magnitude exists across the wall of the striated ducts in the cat submandibular gland. With such a great P D the concentration gradient for potassium lumen/interstitial fluid of maximally 10.4 is far from sufficient to give a passive outflux of potassium. As the time course f the positive sodium and negative potassium transient is very much the same, the most reasonable explanation of these transient phenomena seems to be that they are due to low sodium reabsorption and potassium secretion during the transient period compared with the steady state period. Preliminary experiments in which close intraarterial infusion of aldosterone leads to disappearance of th positive sodium and the negative potassium transients seem to support this hypothesis. It is well known from other epithelia that aldosterone affects sodium reabsorbing processes (Leaf 1966)

It is interesting that the positiv sodium transient in the saliva is synchronous also with the negative potassium transient n the venous outflow from the gland. During the period of the positive potassium transient the gland loses potassium and during the period of the negati e potassium transient the potassium content is built p again. It is evident that the gland takes up potassium from the blood during the period of the negative potassium transient, but it is not so certain whether it also takes p potassium from the precursor saliva. The minimal potassium concentration d ring the negative potassium transient period is very close to the concentration of potassium in the primary secretion from the rat submandibular gland (Young and Schögel 1966) This is consistent with the hypothesis that potassium is not taken up from the precursor saliva, but that the potassium secretion in the duct stem can be abolished during the transient period.

A w y of explaining the connection between the positiv sodium transient and

negative potassium transient in the venous blood would be to suggest that the potassium accumulating process, which is enhanced during the negative transient period, somehow inhibits the sodium reabsorbing process in the duct system, but it is also possible that it is the intracellular potassium concentration which regulates the rate of the sodium reabsorption and potassium secretion.

The sodium transient phenomenon is so complex that not all our findings are easily explainable on the basis of our present knowledge. Especially the frequency shift transients are difficult to explain. It cannot be excluded that a major determinant of the sodium transport in the salivary duct system is the blood flow through the region of the striated ducts, and that an autoregulatory system as it has been suggested in the kidney (Leyssac 1967) regulating both blood flow and active sodium transport is present in the salivary glands.

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Static Load Length Characteristics of Aortic Strips from Hypertensive Rabbits

By

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Abstract

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Elevated threshold and reduced sensitivity of aortic baroreceptors in hypertensive rabbits (Aars 1968) might be due to changes in the aortic wall, which is known to become stiffer in chronic hypertension. Since resetting of baroreceptors was present when studied few days after development of hypertension, the static load-length relationship of circular aortic strips from the receptor area was investigated in vitro. The strips from the hypertensive rabbits were thicker than normal when stretched by 3 g, and had higher elastic modulus than the strips from normal rabbits. Although not strictly comparable to in vivo conditions, the results suggest that at the same blood pressure, the aortic baroreceptors would be less stimulated in the hypertensive than in the normal rabbit, and that given elevation of mean blood pressure would produce greater increase of baroreceptor activity in the normal than in the hypertensive animal. Thus, the changes demonstrated in the aortic wall after 3—15 days of arterial hypertension may explain the resetting of aortic nerve activity in the hypertensive rabbits.

Studies in hypertensive rabbits have shown a resetting of baroreceptor activity in hypertension, with increased threshold and reduced sensitivity (Aars 1968). Since the physiological stimulus for arterial baroreceptors is the stretching of the vessel wall caused by the pressure and not the blood pressure itself (Hauss, Kreuziger and Asteroth 1949), the characteristics of the wall play a dominant role in receptor activity (Heymans and Neil 1958, Peterson 1962). In chronic hypertension, aorta and other central arteries are known to be less distensible than normal (Karnbaum 1961, Feigl, Peterson and Jones 1963, Greene *et al.* 1966), but simultaneous recordings of baroreceptor activity and dynamics of the arterial wall in the receptor area, allowing comparison between normal and hypertensive animals, have not been made.

The reduction of aortic baroreceptor activity in rabbits (Aars 1968) suggested that changes in the aortic wall could be responsible for the resetting. These rabbits, however, were examined after only 3—15 days of hypertension. The present

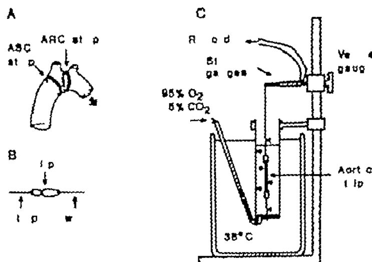


Fig. 1. A. The aortic ar h, with the location of the strips. B. The trip is suspended by means of aneurysm lips. C. The equipment for studying the load-length characteristics of the aortic strips. See text for further description.

was undertaken to find out f the properties of the aortic wall had changed in this short period. The load length characteristics and elastic modulus of the wall were studied in circular strips f aortic tissue f om the receptor areas.

Material and methods

Vernier height gauge (Kanon) and testing hamber containing Krebs'-bicarbonate solution 0.01 M glucose F rchgott 1960) were used for test ng the static load-length characteristics of aortic strips (Fig. 1). 20 ml of concentrated electrolyt solution as mixed with 38 ml 1.3 % N HCO₃ and 0.45 g glucose in 180 ml distilled ter and aerated with 5 % CO_2 in O_2. The strips wer suspended from the horizontal beam of the Vernier gauge and the load resulting from stretching of strip was measured by tw ire strain gauges glued to the beam and re orded on Leeds nd Northrup potentiometric recorder. Deflection of the beam was 0.3 mm with 100 g load, and the height gauge allowed accurate readings of 0.1 mm. The testing chamber as placed in large water bath, here the temperature was maintained at 38 C by immersion heater th electrical sti rer.

Seven normal and seven hypertensive rabbits er used in the experiments. They ere of the same breed, of both sexes and w th mea body eigh 3 200 g in both groups. Arterial hypertension had been induced by wrapping one kidney in silk and performing contralateral nephrectomy fou eek la er. Activit of the aortic nerves as ecorded 3—15 day after nephrectomy. Ca otid arterial pressu as measured with Statham transducer. Prior to recording of nerv ac n ty mea arterial presse was 200/139 mm Hg in the hypertensive rabbits, whereas he normal rabb ts had mean pressure of 132/100 mm Hg.

The animals er anesthet zed with hloralose 30 mg/kg and urethane (0.75 g/kg). Following recording of aort ne ac ivit (Aars 1968 the animals ere bled to death and the aorta emoved. The aor ar h di ected free leared of dventitia, and opened along the lesser curvature. Ci ula st ps f om he aortic arch (ARC and f om the distal part of the ascending aorta (ASC F 1 er excised w th sharp azor blades mounted 1.3 mm apart. One grossly sclerotic strip d arded. I three ases the distance between the tw arteries leaving the arch was so sl rt f r strips to be cut. During preparation, the strips ere kept moist by Ringe solution at oom emperatu. Modified aneurysm clips were ttached to

The stock electrolyte solution as m de of 82.6 g NaCl 4.22 g KCl, 3.36 g $CaCl_2$, 1.94 g KH_2PO and 3.5 g MgSO 7 H_2O 1 000 ml aq. dest.

both ends before the strips were transferred to the testing chamber (Fig 1) Eleven strips from the normal rabbits and 13 from the hypertensive rabbits were examined. The strips were loaded with 3 g for 1 1/2 to 3 hrs (mean time 147 min for strips from hypertensive rabbits, and 133 min for strips from normal rabbits) in order to reach steady length (Aström 1964) Each strip was then fastened to hook t the bottom of the chamber and tested

The strips were initially stretched to length producing a load of 15 g for 2 min, followed by return to length corresponding to 3 g. After 1 min with this load, the length of the strips between the clips was measured and defined as the basic length (L_0)

The strips were then stretched, by 2 mm steps t low elongations, and 1 or 0.5 mm at longer to maximum load of 15—20 g and then stepwise relaxed. 60 sec were allowed for reaching steady state at each elongation. At least three runs were made with each strip. At the end of the experiments, noradrenaline was sometimes added to the bath to check the reactivity of the strips.

Rough estimates of the thickness of the strips were obtained with Vernier calipers after removal of the load, and from weighing after subsequent storage in formol saline. These estimates were supplemented by calculations of wall thickness in strips from other normotensive and hypertensive rabbits, based on weight and length with 3 g load. A density of 1.06 was employed (McDonald 1960) The thickness of strips loaded with 3 g was used in subsequent calculations of elastic modulus. Unfortunately the weights of hypertensive ARC strips were not recorded. Mean thickness of hypertensive ARC strips at basic length was therefore calculated by applying the ARC/ASC ratio found for mean thickness of unloaded hypertensive strips.

The relationship between blood pressure (P) tangential tension (T) and radius (R) is given by Laplace formula

(1) $$T = P \times R$$

Since the circumference of the intact vessel (L_c) is 2 R, the blood pressure corresponding to the measured load and length of strips can be calculated as

(2) $$P = \frac{\text{load}}{\text{width of strip}} \times \frac{2}{L_c} \times 0.74 \text{ mm Hg}$$

where 0.74 is the conversion factor from g to mm Hg the width and length are given in cm, the load in g. L_c was taken as mean basic length L_0 plus 0.2 cm t correct for the parts of the strips outside the clips, and the load as the mean load at each elongation.

Young modulus of elasticity was calculated as the incremental modulus

(3) $$E = \frac{\Delta g \times L \times 981}{A \times \Delta L} \text{ dyn/cm}$$

where 981 is the conversion factor from g to dyn, A the cross-sectional area of the strip and ΔL the increase from previous length L caused by the increment of load Δg.

Comparison of elastic moduli required basic length where the load per unit cross-sectional area was equal. The mean g/cm² in the group of normal ASC strips at 3 g was chosen as the standard, and the loads, lengths and cross-sectional areas producing the same mean g/cm² in the other groups of strips were calculated The calculations were based on data from the load-length diagrams, and the strips were assumed to stretch isovolumetrically, i.e. Poisson ratio of 0.5 was employed (McDonald 1960 Bergel 1961) The resulting corrections were applied to all individual strips

Corrections ere not made for reduction of cross-sectional area of the strips with further stretch

Results

Adaptation to a new length was complete after 60 sec at mall and medium elongations even at the greatest elongations most of the relaxation had taken place in this time Fig In this respect, no differences were found between the groups. The stress relaxation was not further studied, nd the loads recorded t various elongations of strips were considered representative of a steady state

The preparations changed ery l ttle after the initial pre loading and pre-stretching procedures. From the third run, the load-length relationship was practically unchanged F g 3) and onl measurements obtained during this run were

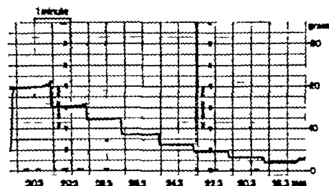


Fig. 2. Original recording of load (ordinate) at various elongations of circular aortic strip from a hypertensive rabbit.

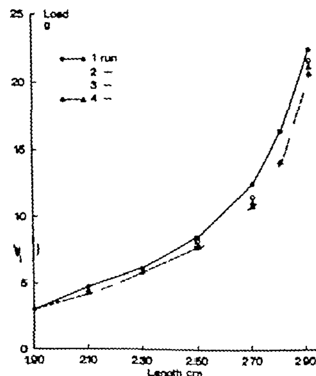


Fig. 3. Load-length diagram of strip from ascending aorta. Hypertensive rabbit. The results are plotted from 4 subsequent runs of stepwise increase in length.

Mean wall thickness was significantly higher ($P<0.02$) in ASC strips from hypertensive rabbits (0.050 cm ± 0.13 (S.D.) n = 8) than from normal rabbits (0.036 ± 0.07 n = 10). Normal ARC strips (0.026 cm ± 0.05 n = 5) were thinner ($P<0.02$) than normal ASC strips. Mean thickness of hypertensive ARC strips was 0.040 cm (see Methods). Mean basic length (with 3 g load) was also increased in strips from hypertensive rabbits (Tables I and II) but comparatively less than the thickness. This resulted in a lower mean radius/wall thickness ratio in aorta from hypertensive rabbits (ASC 7.0, ARC 8.1) than from normal rabbits (ASC 9.1, ARC 11.8).

TABLE I Load t various elongations of strips from ascending aorta in normal nd hypertensi rabbits, nd the basic length (L_0) of the strips

Length of strips $L/L_0 \times 100$	100 %	112 %	120 %	128 %	136	144 %	152	L_0 mm
Normal rabbits								
Rabbit 439	2.9 g	4.2 g	5.7 g		7.4 g	10.0 g	16.8 g	18.0
440	3.0	4.1	5.5	7.0 g	8.9		12.4	19.6
441	3.0	4.2	5.6		7.2	9.5	13.5	18.5
444	2.9	4.6	6.4		8.6	12.2	18.3	18.2
519	3.2	5.0		7.2	8.8	11.3	16.3	14.2
194	3.1	4.6		6.1	9.0	11.1	17.7	16.3
mean	3.0 g	4.5 g	5.8 g	6.8 g	8.3 g	10.8 g	15.8 g	18.6
S.E.	0.05	0.14	0.21	0.34	0.33	0.48	0.97	0.4
Hypertensive rabbits								
Rabbit 437	3.0 g	4.4 g	5.9 g	8.0 g		11.2 g	21.3 g	19.0
438	3.0	4.5	6.2	8.4	11.6 g		17.9	20.5
505	3.2	4.5	6.3	8.6	12.1	15.2	19.3	20.3
514	3.1	4.2	5.7		7.7	12.4	20.3	18.7
515	3.1	4.5	6.2	8.4		14.5	21.4	19.0**
517	3.0	4.1	5.8	8.7	12.2	20.8		19.7
532	3.1	4.3	5.5	7.2	9.6	14.0	17.0	22.6
mean	3.1 g	4.4 g	5.9 g	8.2 g	10.6 g	14.7 g	19.6 g	20.1
S.E.	0.03	0.06	0.11	0.23	0.87	1.36	0.74	0.6
P*				<0.01	<0.05	<0.05	<0.02	<0.05

The elongations are grouped in 8 per cent intervals and the loads are listed at the mean of each step.

Strip shortened during trimming procedures. Length not included in mean length of group.

Significance of difference between the groups tested with Student T-test.

As *the basic length varied, the stepwise elongation of 0.5—1—2* mm produced different relati increases of length. The facilitate comparison of the strips, the loads resulting from elongation were related to steps of 8 per cent increase in length and plotted at the mean of each interval (112, 120 etc.) The results of the load-length studies in ASC and ARC trips are presented in Tabl I and II ARC and ASC strips ga equal results up to 136 per cent of basic length. From 144 per cent ARC strips were less distensible than ASC strips. Strips from normal and h pertensive rabbits showed similar and nearly linear relationships between length and load t small elongations, but from 128 per cent of basic length the strips in the h per tensive group showed increasingly higher resistance to stretch than normal (Table I and II)

TABLE II Load t arious elongations of strips from aortic arch in normal and hypertensive rabbits, and the basic length (L_0) of the strips

Length of strips L/L_0 100		100	112	120 %	128	136	144	15.. %	L_0 mm
Normal rabbits									
Rabbi	439	3 0 g	4.5 g	6.3 g		8.8 g	14.0 g	22.9 g	17 4
	440	3 0	4 0	5.6	7.5 g		12.3	24 0	18.5
	44	3.0	4 5	6 2		8 6	15 0	20.5	18.2
	444	3 0	4 6		6.5	9 1	11 0	15.0	16.2
	19		4 2	5.7		8.4	13.2		16.4
mean		3.0 g	4.4 g	6.0 g	7.0 g	8 7 g	15.1 g	20 6 g	17.3
S E.		0	0 11	0 18	0.50	0 15	0 70	2.01	0.5
H pertens bbits									
Rabbi	43	3 3 g	3 1 g	7 3 g		10.5 g	16.6 g	28.3 g	17 6
	438	3 3	4.5	5 9	7.5 g	9.9	14.2		12.5
	0	2 8	4 5	6.5		11 6	19 0		18.5
	514	3 0	4 5	6 4		8.9	14 7		17.2
	1	3 0	4 4	6.5	9 4	12.2	20.5		19.3
	1	8	4 4	6 4		10.5	16 5	22.1	17.5
mean		3 0 g	4 5 g	6 3 g	8 5 g	10 6 g	16 9 g	.3 2 g	18.1
S.E		0 10	0 12	0 19	0.93	0 48	0.98	3.10	0 4
P*				< 0.001		< 0 02	0.07	0.30	0.15

The l ng on ar grouped in 8 per en intervals and the loads are listed at the mean of ea h ep

S p le rtened during trimming procedures Length not included in mean length of the group.

Sign fic d differenc between the groups tested ith S udent T-test.

Since the rtic all n hypertensive rabbits was thicker each unit of wall thick ness was les t et hed than in normal strips by a load of 3 g This was compensated for by finding th c rrected ba ic length (L) where mean g cm² was similar in all groups of trips nd equal t the g cm² existing in normal ASC strips w th a 3 g load. Equal elat e ncreases of length will then produce uniform stretching of each unit of the wall n all trips When plotted n this way strips from hypertensive rabbits had a higher load th n the normal strips at corrected basic length. and at all subsequent loads the normal trips tret hed more than the others (Fig. 4) In Fig 5 mean load is expressed n terms of calculated pressur and plotted against the relative increase of corrected mean basic length (L) for ASC strips from normal and hypertensive animals

The elastic modulus in normal ASC and ARC strips was almost unchanged up to 120 per cent of corrected basic length I and from 128 per cent rose steeply with

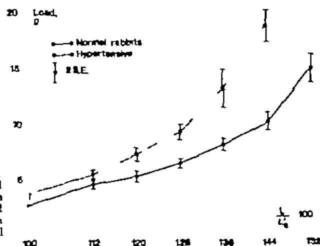


Fig 4 Load-length diagram of 11 ASC and ARC strips from normal rabbits and 13 similar strips from hypertensi rabbits. I Fig 4 5 nd 6, L₀ is the corrected basic length where tension (g/cm) is equal in all the strips.

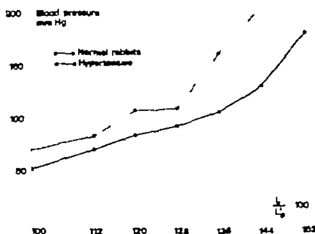


Fig 5 Approximate relationsh p between longation of strips from ascending aorta nd rterial blood pressure, derived from Eq 2

f rther stretch (Fig 6) The elastic modulus of strips from hypertensive rabb ts was equal to that of normal strips at small elongations, but above normal from 120 per cent relative elongation.

All trips tested contracted when noradrenaline was added to the bath at the end of the experiments.

Discussion

The load-length relationship of aortic strips from normal and hypertensi e rabbits differed, whether compared from a length determined at equal loads or at equal relati stretching of each unit of cross-sectional area of the strips (Fig 4) Th dif ference was due to the strips from hypertensive rabbits being thicker and less distensible per nit cross-sectional area than normal strips (Fig. 6)

The period of loading w th 3 g prior to testing of the strips varied, but wl

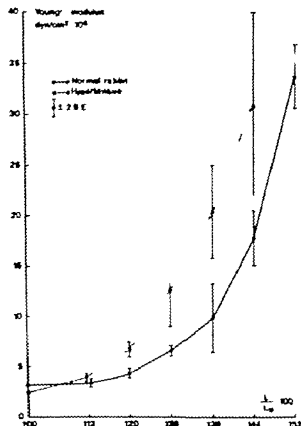


Fig 6 Incremental static lastic modulus (Young modulus, Eq. 5 t arious elongations of the aortic strips. Thirteen strips from hype tensiv rabbits, and 11 strips from normal rabbits. The two curves are significantly different from 112 per cent upwards ($P<0.02$ Students T-test)

sistent bias and with only insignificant difference between mean duration in the o groups Ast om (1964) found that loading with 2—4 g caused aortic strips to stretch 30—40 per cent immediately and to reach a constant length within —3 hrs.

The ela tic modulus of normal strips was within the range observed by others (reviewed by McDonald 1960) Bergel (1961) in pressure-volume studies, at 100 mm Hg found 4·3 10^6 d n cm² in thoracic aorta in dogs, as opposed to the 7.5× 10^6 found in human thoracic aorta by Learoyd and Taylor (1966) with the same technique. The comparison of elastic moduli from a length corresponding to equal initial g cm² in th trips is based on the assumption that the weight-bearing capacity per unit of cross-sectional area was the same in strips from normal and hypertensive rabbits near 5 g Th diff ence at higher relative elongations between the two curves in Fig 6 w ll probabl not be significantly educed even if the assumption is not completely correct The mall ariations of load and elastic modulus at each relative elongation n F g 4 nd 6 indicate uniform composition of the wall in ascending aorta and the aortic h This is in accordance with the observed similarity in the two areas in ont t f lastin and collagen (Harkness, Harkness and McDonald 1957 Wolinsk d (l sow 1964) Apter Rabinowitz and Cummings (1966) however found a small d fference

Correlation with in vivo conditions is more difficult, and can only be approximate. Whereas a pressure rise in aorta results in circular stretching and thinning of the wall, with minimal longitudinal elongation, increase in length of the circular strip will cause reduction both in thickness and width of the tissue. Furthermore, the precise circumference of the wall, essential to calculation of blood pressure, was unknown. Some strips were shortened during trimming procedures, and the parts of the strips outside the clips varied in length. When plotted against relative increase of corrected basic length blood pressure could be directly related to the stretching of each element of the aortic wall in normal and hypertensive rabbits. It is evident from Fig. 5 that any blood pressure will stretch the elements of the wall less in hypertensive than in normal rabbits. Unless the sensitivity of the aortic stretch receptors had increased, the different pressure-length relationship would result in resetting of baroreceptor activity in the hypertensive rabbits.

Fig. 5 shows that the strips were exposed to the same range of pressures as the aortic baroreceptors (Aars 1968). Measurements of the diameter of the intact ascending aorta in rabbits have shown that a 28 per cent increase in diameter is equivalent to a rise of diastolic blood pressure from 50 to about 150 mm Hg (unpublished results). 128 per cent elongation of normal strips corresponded to an increase of calculated blood pressure to about 90 mm Hg (Fig. 5). The calculated blood pressures might accordingly be too low at high elongations.

The circular stretching of the aortic wall at a given pressure is determined by the radius wall thickness ratio. A wall with a low ratio will stretch less than a wall with a high ratio in response to the same pressure. The reduced radius wall thickness ratio in hypertensive rabbits is the physical basis for the difference between normal and hypertensive animals around basic length of strips illustrated in Fig. 5. Karnbaum (1961) measured the circumference and thickness of aorta at zero pressure in humans at autopsy. Calculations from his data show no differences in the radius wall thickness ratio in proximal ascending aorta in comparable age groups, but the ratio was reduced in the distal part of descending aorta from older hypertensive patients. In femoral arteries of hypertensive and normal dogs, Feigl, Peterson and Jones (1963) did not find significant changes in this ratio.

The present investigation has shown that only 3—15 days after the development of hypertension, significant changes had occurred in the aorta. The strips from hypertensive rabbits had higher elastic modulus than strips from normal rabbits, and were thicker with reduced radius/wall thickness ratio at basic length. The net result was that they were less distensible than normal at all pressures. This is very similar to the results of pressure-volume studies in chronic hypertension. The aorta (Karnbaum 1961), carotid sinus (Asteroth and Kreuzinger 1951) and brachial artery (Greene *et al.* 1966) are less distensible in humans with hypertension. Simon and Meyer (1958) found slightly increased volume distensibility in aortas from hypertensive patients, but they allowed the specimens to stretch with a rise of pressure. Six aortas from hypertensive patients showed greater longitudinal elongation than the five comparable aortas from normotensive patients, and their results can

cordingly be compared with those of the other authors. Based on simultaneous measurements of pressure and vessel diameter the femoral arteries in dogs were shown to have increased stiffness owing to changes in the vessel wall material during hypertension (Feigl *et al.* 1963).

Although the present results were arrived at by studying the tissue in steady-state conditions in vitro and their extent of applicability to the intact pulsating aorta is uncertain, the data would suggest that the aortic wall requires a higher pressure in a hypertensive rabbit than in a normal rabbit to produce the same stretching of the wall elements and the same baroreceptor activity. The reduced distensibility would imply a smaller aortic wall expansion, and less increase of baroreceptor activity in response to a given elevation of mean blood pressure. Thus, the changes demonstrated in vitro in the aortic wall would, if valid in vivo, give a resetting of baroreceptor activity similar to that found in hypertensive rabbits (Aars 1968).

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From the Institute of Physiology University of Oslo Norway

Effects of Temperature Changes on the Pressor Response to Acute Alveolar Hypoxia in Isolated Rat Lungs

By

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Abstract

Haavik Nilsen K. and A. Hauge. *Effects of temperature changes on the pressor response to acute alveolar hypoxia in isolated rat lungs* Acta physiol. scand. 1968 73 111—120

The effects of perfusate cooling upon the pressor response to 5 min periods of ventilation hypoxia was tested in an isolated blood-perfused rat lung preparation. The response was found to be markedly and reversibly thermosensitive. Lowering the perfusate temperature from 38° C to 27.5 C abolished the pressor response to the subsequent test of ventilation hypoxia. When, however perfusion was continued at 27.5 C moderate pressor responses to ventilation hypoxia developed. The number of pressor responses which could be obtained to 5 min tests of hypoxia was limited in this preparations. In perfusions carried out at 27.5 C the responses were smaller but 1 1/2 to 2 1/2 times as many, as in perfusions at 38 C. The thermosensitivity of the pressor response could not be correlated with pH changes in the perfusate The findings seem to support the theory of vasoconstrictor agent being released or activated by alveolar hypoxia.

The mechanisms underlying the pressor response to alveolar hypoxia are still not known (Daly and Hebb 1966) This response can be obtained in isolated perfused lung preparations from various species. It has been reported however that this response can be evoked only for a relatively short period of time in such preparations from the dog (Lloyd 1964) and from the rat (Hauge 1968) In isolated perfused lungs and lung lobes of the dog the pressor response to alveolar hypoxia has also been shown to be markedly dependent on perfusate temperature (Daly Ramsay and Waaler 1962, Daly and Hebb 1966, Lloyd 1966b) Daly Ramsay and Waaler (1962) found in isolated perfused preparations of the dog that with initial ischaemic periods of less than 15 min an increase in pulmonary vascular resistance was the predominant response to ventilation hypoxia over the whole temperature range tested (28.5 to 41.5 C) After ischaemic periods of between 15 and 35 min the pressor response was obtained at the higher but not at the lower temperatures. Also the pressor responses increased with increasing temperatures. Lloyd (1966b) reported

that graded degrees of perfusate cooling in his isolated dog lung preparations caused graded reductions in the pressor responses to hypoxia. A complete depression of the pressor response could be obtained by cooling the perfusate in one step from 36 to 26 C. Perfusate cooling also prolonged the time span during which he could obtain pressor responses to ventilation hypoxia. No further comments on these findings were made.

The present study was undertaken in order to analyze more closely the effects of changes in perfusate temperature upon the pressor response to ventilation hypoxia. It was hoped that such an analysis might provide information about the nature of this response and its underlying mechanisms. Isolated perfused rat lungs, which have been shown t give marked and reproducible vascular responses to hypoxia, were used for the tudies.

Methods

A mal d er ch ics. Male albino rats (350—450 g) of a local strain (originall Wistar) were sed T blood donor animals were anesthetized w th ether and exsanguinated by heart puncture. Bet een 18 and 2? ml of blood were obtained, and t this was added 1 ml of solution ontaining 200 of heparin (Nap nr 1337 Apothekernes Laboratorium, Oslo) in saline.

The rat from which the lungs were to be taken was given light introductory anesthesia ith ethe nd then intraperitoneal injection of 4—5 mg/100 g b.w. of pentobarbitone (Nembutal®, Abbot, dilu ed 1 5 in saline). The lung preparation was dissected out and cannulated mainly as described by Hauge (1968). One modification in the procedure was that the lung donor rat as entilated with oxygen (instead of air) during the dissection procedure. The time elapsing between the interruption of the rats ow circulation and start of the perfusion ischaemic period" was between 10 and 11 min in all experiments.

Perf sio The lungs were hanging freely inside thermostatically controlled perspex chamber and the pulmonary ascular bed was perfused with constant olume pulsatile inflow as described by Ha ge 1968). The blood entered the preparation through cannula inserted through the righ entricle into the pulmonary artery and drained into the blood reservoir by w of annula inserted into the left entricle. The tip of the outflow cannula was positioned low he trio- en ricular alv It was not pressed through the valv orifice and into the atrium as as found ha this might cause some obstruction of the blood drainage. The aorta was ligated just outside the heart. The temperature in the blood reservoir was also thermostatically ontrolled. The pulmonary arterial pressure (P A.p.) was followed continuousl ith Statham P 23 Gb pressure transducer connected to Sanborn Model 320 DC amplifier recorder or Sanborn Model 350—1100 DC carrier preamplifier. The P_{O_2} of effluent blood was measured nd recorded continuously with Beckman micro oxygen electrode connected to a Physiological Gas Anal zer (Beckman, Model 160) and from there to the Sanborn Model 320 DC amplifier recorder.

The blood flow ra es used ranged from 7 to 8.5 ml/min in this series of experiments, and was kept constant hroughout each individual experiment. W th this flow the P A.p. aried between 10 and 15 mm of Hg from the outset of each perfusion. Left trial pressure was kept constant in each experiment at level of from 2 to 3 cm of blood.

Ventilation. Positiv pressure entilation frequency of 36/min was carried out after one or two initial gentle inflations. The entilation overflow was recorded continuously with the arrangement described by Konzet and Rössler (1940). The gas mixtures used for ventilation were 1) 8 % CO_2 in ai and 2 2 % O_2, 8 % CO_2 and 90 % N_2 (referred to as "the 2 % oxygen mixture" I tw experiments the CO_2 content was 5 % in both the air and the % oxygen mixture. I order to counteract the tendency of the tidal volume to fall during long lasting perfusions the lungs wer gently inflated 5 ml prior to each test period of hypoxia throughout most of the experiments.

Th temperature of the effluent blood was me with YSI Tele Thermometer (Yellow Springs Instrument Co., Inc. M ... ing and rewarming of the perfusate blood and the ... on were done water I the thermostated water jackets surrounding ... reservoir and

The pH of the effluent blood was measured with the Astrup Micro Equipment (Radiometer type AME 1) The pH-meter was always calibrated for the temperature of the blood to be measured, so that the actual pH could be read directly Triple measurements were always carried out and they agreed within ±0.01 pH units.

Drug injections were carried out into the pulmonary arterial tubing. The drugs used were Adenosine triphosphate (Adenosine 5-triphosphate disodium salt, crystalline from Equine Muscle, Sigma Chemical Co.) and bradykinin (Synthetic Bradykinin, BRS 640 Sandoz A.G) They were dissolved in saline and injected in volumes of 0 4 and 0 1 ml respectively

Results

On the basis of preliminary tests it was decided to evaluate the pressor response to acute alveolar hypoxia at 4 standardized levels, namely at 38 (control) 34.5 31 and 27.5 C. Unless otherwise stated experiments were started with the perfusate temperature set at 38 C and the lungs ventilated with 8 % CO in air From 10 to 23 min after start of perfusion 3-min test periods with ventilation hypoxia were applied at intervals of 7 min. The 2 % oxygen mixture (see Methods) was used as the ventilating gas during these test periods.

The pressor responses to ventilation hypoxia developed according to the pattern described by Hauge (1968) and as shown in the left graph of Fig. 1 The first one or two periods with hypoxic ventilation did not usually cause any pressor responses and are referred to as the non-effective test periods The subsequent tests which did elicit pressor responses will be referred to as "the effective test periods" Intervals between subsequent ventilatory tests were prolonged from 7 to 15 min after the occurrence of the first such effective test period.

The maximal response to ventilation hypoxia was in the present experiments always obtained in the third effective test period, if a temperature level of 38 C was maintained throughout. Thereafter the responses declined, and finally no rise in pulmonary arterial pressure was seen during the 3 min hypoxia tests. Pressor responses caused by injections of bradykinin and ATP were, however then not diminished.

In order to evaluate the effects of cooling upon the hypoxic pressor response the perfusate temperature was lowered during the interval between the second and the third effective test period. Thus the third effective test period, which would normally elicit the strongest pressor response, was carried out during lowered perfusate temperature Before start of the fourth effective test period the temperature was again increased up to 38° C and kept at that level throughout the rest of the experiment. In 3 expts. with such intermittent cooling to 34.5 C the third pressor response was considerably reduced, reaching a level corresponding to only 23 30 and 3? per cent of the second response. In 4 expts. with temperature reduction to 31 C the third response was reduced to 0, 14 17 and 28 per cent of the second one When the perfusate was similarly cooled to 7.5 C no pressor response to ventilation hypoxia was seen during the third effective test period in any of 7 expts. carried out. The results of the total number of tests in two lungs with such perfusate cooling to 34 5 and 27.5 C respectively are shown in the middle and right graphs of Fig 1 Fig 2 illustrates some of the tests in an experiment with perfusate cooling to 27 5 C

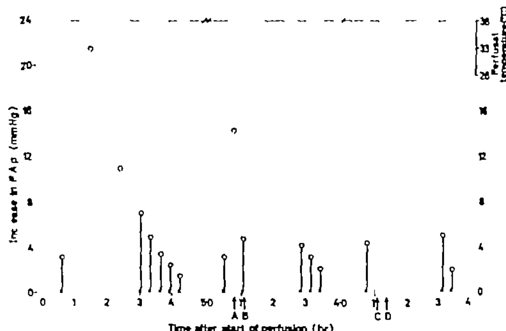
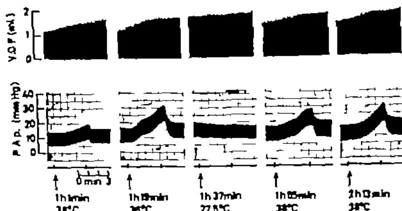


Fig 1 Effects of reductions of perfusate temperature on the pulmonary vasoconstrictor response to acute alveolar hypoxia.
3 perfusion-expts are shown. Left control experiment, temperature 38 C throughout. Middle and right temperature lowered temporarily to 34.5 and 27.5 C, respectively. Temperature reductions were started between the second and the third effective hypoxic test (at A and C respectively and the return to control temperature was carried out between the third and the fourth test period (t B and D respectively) Black squares on the bottom represent 3 min test periods of ventilation hypoxia. Hypoxic gas mixture % O_2, 8 % CO_2 90 % N. Control gas. 8 % CO in air Vasoconstrictor responses (ertical bars) are given as maximum increases in pulmonary arterial pressure (P A.p.) during each test period. Baseline P A.p rose less than 5 mm Hg during the experiments shown in this and the other figures. Left atrial pressure and blood flow through the lungs were kept constant t 2–3 cm of blood and at about 8 ml/min respectivel

A moderate degree of perfusate cooling thus caused a marked reversible depression of the pressor responses to ventilation hypoxia in the present preparation. When the perfusate temperature was maintained at the low level of 27.5 C, we found that the inhibition of the pressor response to ventilation hypoxia was not a permanent one. If at that temperature hypoxia lasted for more than 3 min, a moderate rise in pulmonary arterial pressure regularly appeared. And if as shown in Fig 3. the perfusate temperature was kept at 27.5 C long enough for three 3-min hypoxia tests to be carried out at 15 min intervals a pressor response was always elicited to the second and a stronger one to the third of these low temperatures tests. Uniform results were obtained in 3 expts of this type

In 5 preparations the temperature was kept unchanged at 27.5 C from the outset of perfusion and throughout the experiment, the ventilation tests and the intervals being the same as in the other experiments described. A typical experiment of this type is shown in Fig 4 together with one typical experiment carried out at



F g 2 Effect on the pulmonary asoconstrictor response to lveolar hypoxia of reduction of perfusate temperature to 27.5 C.

Isolated perfused rat lung preparation. Three-min tests with ventilation hypoxia (entilation gases see Fig. 1) were started at arrows and ended at the econd time-marks. Time after start of perfusion and perfusate temperature are given for each test. P.A.p. pulmonary arterial pressure V.O.F entilation overflow A gentle inflation was carried out 3 min prior to each hypoxia test (see Methods)

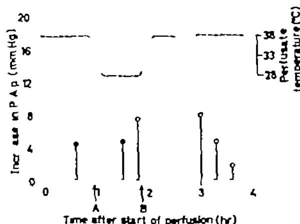
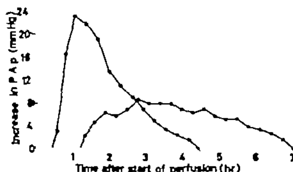


Fig 3 The effect on pulmonary pressor responses to ventilation hypoxia of reducing perfusate temperature to 27.5 C during 5 sequential tests

Isolated perfused rat lung preparation. Tests with ventilation hypoxia carried out and asoconstrictor responses given as explained under Fig. 1 P.A.p. pulmonary arterial pressure.

Fig 4 Pulmonary asoconstrictor responses to repeated periods of alveolar hypoxia at perfusate temperatures of 38 and 27.5 C respectively

T isolated perf sed rat lung preparations Test with entilation hypoxia carried out and asoconstrictor responses indicated as explained in Fig 1 ○ Pressor responses to hypoxia in experiment with perfusate temperature of 38 C. Pressor responses to hypoxia in experiment with perfusate temperature of 27.5° C. P.A.p Pulmonary arterial pressure



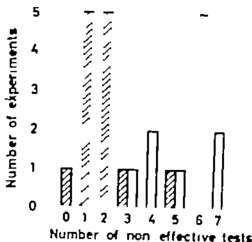


Fig. 5 Number of initial non-effective test periods with alveolar hypoxia in 24 experiments with perfusate temperatures of 38 and 27.5 C.

Results from 24 individual expts. with isolated perfused rat lungs. Test with ventilation hypoxia carried out as explained in Fig. 1. Hatched columns experiments with perfusate temperature of 38 C. Open columns experiments with perfusate temperature of 27.5 C.

38 C. It will be seen that the number of non-effective test periods were higher in the perfusion carried out at 27.5 C than in the perfusion carried out at 38° C. Fig. 5 gives the number of non-effective tests in all the experiments of these two types. The maximum response appeared later and was much smaller in a 27.5 C perfusion than in a 38 C perfusion. The number of responses obtained was, on the other hand, 1 1 2—2 1 2 times as great in 27.5 C perfusions as in 38 C perfusions (Fig.

When a response from a 27.5 C expt. was compared to an equally sized response from a 38 C expt (Fig 6) there were no apparent differences in the intervals be-

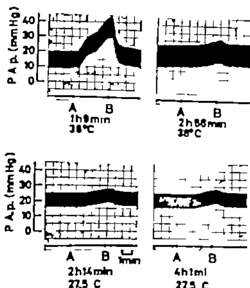


Fig. 6. Pulmonary arterial pressure responses to alveolar hypoxia in two experiments with perfusate temperatures of 38 and 27.5 C respectively.

Upper two tracings from one isolated perfused rat lung experiment with perfusate temperature of 38 C, lower two tracings from another one carried out at 27.5 C. Tests with ventilation hypoxia carried out as explained in Fig 1. The left two tracings show the third effective test period in each of the two experiments, the two right ones show the ninth effective test periods. Temperature and time after start of perfusions given for each test. P.A.p.: pulmonary arterial pressure.

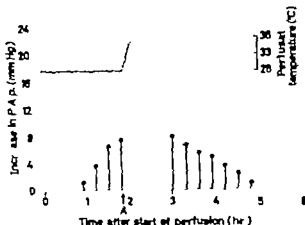


Fig. 7 The effect on pulmonary vasoconstrictor response to ventilation hypoxia of elevating perfusat temperature from 27.5 to 38 C.

Isolated perfused rat lung preparation. Perfusate temperature was kept at 27.5 C from the outset of perfusion until between the fourth and the fifth effective test period, at A, when it was elevated to 38 C. Test with ventilation hypoxia carried out and vasoconstrictor responses given as explained in Fig 1 P.A.p. Pulmonary arterial pressure.

tween start of ventilatory hypoxia and start of the rise in pulmonary arterial pressures. Nor were there apparent differences between the slopes of these rises. The recovery slope on changing back to ventilation with the air-mixture was, however distinctly steeper t 38 C than at 27.5 C.

In further experiments the temperature was kept at 27.5° C from the *outset* of perfusion until the fourth response had been obtained. During the subsequent interval the temperature was increased to 38 C and kept at that level throughout the rest of the perfusion. The fifth response was then in all 3 expts. of the same magnitude as the maximal responses found in perfusions where the temperature had been maintained at 38 C *from* the outset (Fig 7)

The pressor responses to hypoxia in isolated dog lungs are sensitive to pH variations of the perfusate in that an increase of pH within a certain range cause a reversible decrease of the responses (Lloyd 1966a) Cooling of blood is known to cause an increase of pH (Rosenthal 1948) The effect of lowered temperature might therefore to some extent be due to an increase in pH Cooling from 38 to 27.5 C had previously been found to cause an ncrease in the pH of our perfusate of about 0.06 pH unit, from about 7.18 to about 7.24 In order to evaluat this effect the pH of the rat blood perfusate was increased by decreasing the CO_2 content of the ventilation gases from 8 to 5 during tw 38 C perfusions. The pH of the perfusat was in both experiments increased by 0.2 pH unit, from 7.18 to 7.38 and from 7.17 to 7.37 respectively This had, however no apparent effects *upon th* responses to hypoxia.

In order to evaluate possible temperature ind ced changes in respons eness to vasoactive drugs, ATP and bradykinin were in 4 expts. injected at 38 31.5 31 nd 7.5 C respectively As shown in Fig 8 the size of the pressor response t 10 µg of bradykinin was hardly affected by perfusat cooling The pressor resp nse to ATP jections, however wa markedly thermosensitive. The off-effect afte i both drugs took much longer at low temperatures than at 38 C

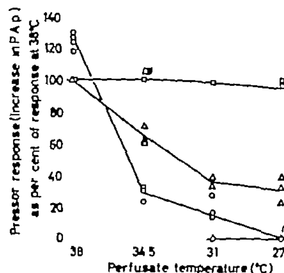


Fig 8. Effect of perfusate temperature on the pulmonary vasoconstrictor responses to acute alveolar hypoxia and on responses to injected standard doses of bradykinin and ATP

Isolated perfused rat lungs. Temperature reduction during tests with ventilation hypoxia carried out as explained under Fig 1 and in text. The points represent the hypoxic pressor response to the third effective test period as per cent of the response to the second one. In each experiment perfusate temperature was reduced from 38 C to the chosen level between these two tests (see Fig 1). For the drug actions the mean value of two responses obtained in one expt. at each chosen temperature level is given as per cent of the mean value of two responses obtained at 38 C in the same experiment. Figures in bracket give number of test results at corresponding points in the graph. O hypoxia, Δ ATP (200 μg in 0.4 ml) □ bradykinin (10 μg in 0.1 ml)

Discussion

The pressor response to acute alveolar hypoxia in this lung preparation is markedly *thermosensitive when compared to the responses to the injected pulmonary constrictor agent* bradykinin. This is in agreement with observations of Lloyd (1966b) using with isolated perfused dog lungs. He found that pressor responses following addition of adrenaline and serotonin to the perfusate was nearly unchanged by perfusate cooling from 36 to 26 C. These findings indicate that the effect of cooling upon the alveolar hypoxic pressor response is not mainly due to a reduction in contractility of the vascular smooth muscle. When in our experiments hypoxic pressor responses of equal maximal size, but obtained at high and low temperature respectively were compared, no significant difference in the rate of pressure rise could be detected. The rate of vascular smooth muscle relaxation subsequent to a con*striction was in our preparation slower at low temperatures than at high* temperatures This is what would be expected according to the work of Rao and Singh (1940) and of Bohr *et al* (1962)

Constrictor responses obtained with ATP differed from those obtained with bradykinin in that they showed a higher degree of sensitivity to temperature changes, being more like the pressor response to hypoxia. The significance of this is not known.

The intrinsic mechanisms for the vasoconstrictor effect of acute alveolar hypoxia is not known. The low alveolar P_{O_2} could act directly on the smooth muscles in the pulmonary arterioles, as these vessels lie close to alveoli (Lange and Hecht 1936, Sobol *et al.* 1963 Jameson 1964 Smith and Vane 1966) This would, however be a unique behaviour of the vessels involved since to our knowledge no vascular smooth

muscle preparation, whether taken from the lungs or from systemic vascular beds has been reported to respond with constriction to hypoxia. Lowering of the oxygen tension in the perfusate or in the organ bath solution has invariably been found to give relaxation in such preparations (Smith and Cove 1951 Lloyd 1967) This is so also for isolated pulmonary vessels of the third order size (Lloyd 1967) indicating that the constriction seen *in vivo* might be mediated through some indirect mechanism. The hypothesis most favoured at present seems to be that the hypoxic pressor effect in the lungs is brought about by the release or activation of a pressor substance originating from the lung tissue itself (Barer 1966 Robin *et al.* 1967) Although not conclusive the results reported here seem to support such a humoral transmitter theory

Liljestrand reported in 1958 that in isolated perfused cat lungs the pressor response to ventilation hypoxia was closely correlated to a lowering of the perfusate pH, suggesting H ions, from increased lactic acid production in lung tissue, as the active agent. The depressive effect of cooling on the response could then be caused by a reduction in the hypoxia-caused increase in local H -concentration. In our preparation a pH elevation of about 0.2 units, brought about by reducing P_{CO_2} in the ventilation gases, had no significant effects on the magnitude of the pressor response to ventilation hypoxia. Lloyd (1966a) has found, however that to cause a reduction of the pressor response to ventilation hypoxia in isolated, perfused dog lungs, pH must be elevated to alkalotic levels (above 7 40) whereas the highest perfusate pH level obtained in this series of experiments was 7.36. The pH increase of only 0.06 units which in our perfusate was caused by cooling from 38 to 27.5 C was also apparently fa too small to explain the temperature induced reduction in the hypoxic pressor response

The most thermosensitive links in the chain of events which cause the vascular smooth muscles to contract during hypoxia will determine the magnitude of the response after cooling These links may be in th metabolism of a pressor agent, either its production, its activation or its inactivation, or in processes responsible for liberation of the agent from extravasal tissue

When a perfusate temperature of 27.5 C was used from the outset and throughout the experiment, the number of hypoxic responses which could be evoked was bout doubled, whereas the size of the individual responses was reduced. This might suggest the in ol ement of a metabolic process, limiting the amount of transmitter substance being produced and made ccessible to the vascular smooth muscle within each test periods.

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From the Department of Anatomy University of Helsinki, Finland

Distribution and Intensity of Monoamine Oxidase Activity in the Mammalian Duodenum

By

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Abstract

PENTTILA, A. *Distribution and intensity of monoamine oxidase activity in the mammalian duodenum.* Acta physiol. scand. 1968. 73 121—127

The quantity and localization of monoamine oxidase (MAO) activity was studied in the duodenum of eight mammalian species.

There were great differences in the enzyme activity between various species. In the guinea pig the rabbit and the mouse MAO activity was 6—9 times as high as that in the rat, the cow the pig and the horse. The sheep differed significantly ($P < 0.001$) from all other species. In the pig, the activity of the muscular layers was about third of that observed in the united mucous and submucous layers.

Histochemically the epithelium adjacent to the intestinal lumen showed strong enzyme activity in all the species but the cow the sheep and the rat. The Brunner glands and the muscular coats exhibited moderate or weak reaction. The serosa and mucous muscular layer were negative.

The results are compared with those made by the present author on the distribution of 5-HT and the enterochromaffin cells in the same species. There is no correlation between chemically estimated MAO activity and 5-HT content in the duodenum. The histochemical distributions of the enzyme activity and 5-HT are also different.

Monoamine oxidase (MAO) is of great importance in the physiological inactivation of biogenic monoamines (Blaschko 1952, Davison 1958) This enzyme is widely distributed in mammalian tissues but its activity varies greatly in different organs of the same species and in analogous organs of different species (Langemann 1944 Davison 1958) Epps (1944) observed chemically a high MAO activity in the gut. In a histochemical study Eder (1957) concluded that epithelial MAO is protective barrier to monoamines formed in the gut lumen.

The gut of all the mammalian species contains also large amounts of biogenic amines, especially 5-hydroxytryptamine (5-HT) (Erspamer 1953 Klingman *et al* 1964) In the gastrointestinal tract of most species, the concentration of 5-HT is highest in the duodenum (Erspamer 1961) It is largely bound by specific enterochromaffin granules (Baker 1958) Klingman *et al.* (1964) found no correlation between 5-HT and norepinephrine MAO and dopa-decarboxylase activities in rat gastrointestinal tract.

The present author (Penttilä 1966) found marked species differences in the 5-HT content of the mammalian duodenum. Because 5-HT is known to be a good substrate for MAO activity (Blaschko 1952, 1957) and especially because no studies have been as yet carried out on the histochemical correlation of MAO with 5-HT or with the number of enterochromaffin cells in the digestive tract, it seemed to be of value to determine this enzyme activity in the same species which have been studied for the 5-HT distribution (Penttilä 1966)

Material and methods

Fresh pieces were taken for histochemical and quantitative studies from the proximal part of the duodenum just caudal to the pyloric ring in the sheep the cow the pig the horse and the rabbit. hereas in the guinea pig, the mouse and the rat the first third of the duodenum was homogenized for quantitative studies. The laboratory animals were killed by blowing on the head the pigs by electrocution and the other domestic animals by shooting on the head. The specimens ere obtained from laboratory animals immediately and from domestic animals as quickly as possible (10 to 20 min) afte bleeding. They were washed several times in saline and cooled in closed glass bottle kept in ice for transporting to the laboratory. Tissues dried by blotting were eighed, pooled and homogenized in a homogenizer (Ultra-Turrax Type 18/2 Janke & Kunkel KG Staufen) with about tenfold volume of cooled distilled water. The enzyme determination was always made within one hour after killing the animals.

MAO activity as determined according to the principle of Weissbach *et al.* (1960) as modified by Krajl (1965) for fluorometric use. Kynuramine (Kynuraminedihydrobromide, Sigma Chem. Comp was included as substrate and ppropriate standards (4-hydroxy-chinolin trihydrat Fluka AG) and blanks were carried through the entire procedure. The optimal activation peak was 315 mm and the fluorescence intensity was read at 380 nm (*uncor* ected instrumental alues) in an Aminco-Bowman spectrophotofluorometer. The protein content of the homogenates was determined by the biuret reaction using bovine serum albumin as standard Cornall *et al.* 1949)

The method of Glenner *et al.* (1957) was used for the histochemical localization of MAO in fresh cryostat sections (10 μ). Tryptamine (Tryptamin hydrochlorid, Fluka AG) ed substr and isonicotinyl-2-isopropyl hydrazine phosphate (Marsilid, F Hoffmann-Roche & C as *selective inhibitor* for MAO activity. The *final* inhibitor concentration *was* 2×10^{-4} M. Controls were tested without substrate.

Results

1 Qua tita i tud

The level of MAO activity in the duodenum of different species are presented in Table I. The enzyme activity of the whole duodenal wall was especially high in the guinea pig and the rabbit. The rat, the cow the pig and the horse showed the same rate of action whereas in the sheep MAO activity was the smallest and differed significantly $P<0.001$) from all the other species investigated.

In the cow and the pig the external and internal muscular layers were dissected separately from the mucous and submucous layers and the separation was checked histologically. In the pig the activ ty of the muscular layers was significantly ($P<0.001$) lower than that of the un ted mucous and ubmucous layers, whereas this difference was not so clear n th cow duodenum (Table I)

2. Histoch mical stud s

Serosa In none of the eight species investigated was there any MAO activity in the serous coat of the duodenum.

Table I. MAO activity of the duodenal tissue of several mammalian species. In all the species enzyme activity is expressed per whole duodenal wall, in the cow and pig also per muscular and mucous-submucous layers. Activity is given as μmoles 4 HOQ formed per gram of wet weight of tissue and per g of protein per hr at 37° C. Values in parentheses indicate the number of tissue samples analyzed. Means and standard deviations

Species	Activity (μmoles 4HOQ/g/hr)			Activity (μmoles 4HOQ/g protein/hr)		
	Avg.	S.D	Range	Avg	S.D	Range
Sheep (12)	1.25	0.24	1.01— 1.79	10.0	1.59	7.49— 13.0
Cow (12)	2.78	0.47	1.91— 3.47	22.5	6.10	14.2 — 34.2
Pig (14)	2.54	0.67	1.39— 3.60	21.8	6.40	11.2 — 34.1
Horse (8)	2.09	0.77	1.18— 3.20	17.5	6.60	8.41— 27.4
Guinea Pig (8)	17.6	3.20	13.9 —23.3	165.	27.9	137 —220
Mouse (10)	12.8	1.85	10.8 —16.6	115.	20.9	77.0 —198.
Rat (10)	3.66	0.44	3.14— 4.56	31.7	9.80	25.4 — 45.5
Rabbit (6)	16.1	54.2	10.4 —26.1	169	60.6	100 —241
Cow Muscular layers (8)	2.33	1.06	1.08— 4.19	22.7	9.76	7.6 — 35.2
Cow Mucous-submucous layers (8)	3.35	0.81	2.57— 4.78	29.4	7.05	17.6 — 37.5
Pig Muscular layers (6)	1.56	0.19	1.36— 1.87	15.7	4.99	7.30— 19.8
Pig Mucous-submucous layers (6)	4.32	0.63	3.32— 5.13	37.2	6.91	33.0 — 50.0

External and internal muscular layers In these layers the enzyme activity was usually weak (Fig 1 and 2) In contrast to the other species investigated, the reaction of the muscular layers was more intense in the cow the sheep the horse and the rat than that of the mucous membrane In the cow and the sheep, the external muscular coat was more reactive than the internal one and the reaction was localized as fine blue longitudinal fibrils. In contrast to the other species, MAO activity was weak or negative in the muscular layers of the guinea pig and the mouse The colour of the reaction product in the muscular layers varied from purplish to blue depending on the reaction time.

Especially in the domestic animals the ganglion cells in the Auerbach and Meissner plexus showed a moderate MAO activity and the nerve cells were easily distinguished among other tissue components (Fig 4)

Submucosa The cytoplasm of the cells in the Brunner glands exhibited weak or moderate enzyme activity in the domestic animals. In the rabbit these cells were easily distinguished from the inactive submucous connective tissue as a narrow strip beneath the reactive mucous membrane (Fig. 2) In the sheep, in contrast to the other species, the enzyme activity was weak or moderate in the cells of the Brunner glands deep in the submucosa, whereas the enzyme activity of the glandular cells decreased towards the mucous membrane. In the other laboratory animals, the enzyme activity of the Brunner glands was weak or negative as were also the cells of the Lieberkühn crypts. The nuclei were always negative.



Fig 1

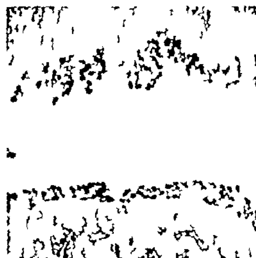


Fig 2

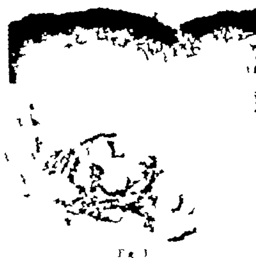


Fig 3

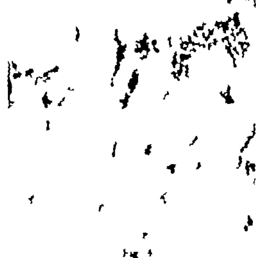


Fig 4

Fig 1 4 M oam ne oxidase of the duodenum

Fig 1 Guinea pig Note h on zyme activit be pithelium sheet al he mucous membr ne M sc la I al mos neg nd MAO acti t low l of the Laeberkuh p 3

Fig 2 Rabb A the n p g he musc la la er nd the rypt of Lieb kuhn are weakly stained H B he B se la ds exhib st ong reaction 5

Fig 3 Pig Note he ac er ty the su fac pi heli m The mos eminent activ t is the spl n he estinal l me 340

Fig 4 Horse The l l l s ll of he Aue ba l ple is re l l d stinguished among the w kl ned la l 25

Mucosa In this l er ther g t ariations n the MAO t t l t een dif ferent species. In th pig th h th gu nea p g th bbit and the ase a strong MAO activit w exhib t l l tl pith l al cells (F g 3 wherea the reaction was weak in th ow nd k neg t the sheep nd the rat. I the

latter two species, the mucous membrane showed a weak or moderate non-specific brown precipitate with longer incubation times.

The epithelial cells all over the villi showed a strong enzyme activity. The staining was strongest adjacent to the intestinal lumen and it weakened clearly towards deeper parts of the villi. The nuclei were without reaction and the goblet cells were negative.

In the guinea pig, the rabbit, and the mouse, the enzyme activity of the intestinal glands weakened clearly towards the basis of the mucous membrane. On the other hand, in the horse, the pig, and the cow the glandular cells of the mucous membrane beneath the surface epithelium showed a uniform weak reaction.

The muscularis mucosae, which was very easily seen in the domestic animals, exhibited no MAO activity. The reticular cells of the lamina propria showed a negative or very weak MAO activity in all the species investigated.

No MAO activity was seen in the presence of 2×10^{-4} M Marsilid as an inhibitor or in the absence of the substrate.

Discussion

The results in the present study showed that there are great differences in the duodenal MAO activity of different mammalian species. This is in agreement with the chemical studies by Davison (1958), Weiner (1961), Ganrot *et al.* (1962) and Levine and Sjoerdsma (1962) in various mammalian tissues including the gut. The relative rate of the deamination of monoamine by MAO varies greatly and the source of the enzyme and the mode of preparation both have an effect on the enzyme activity (Fletcher *et al.* 1966). Therefore, the studies are not directly comparable to each other. Thus, Davison (1958), using manometric MAO determination, found no activity in the rat intestinal tissue. Similarly Koelle and Valk (1957) observed no activity determined manometrically or histochemically in the ileal muscular layers of the rat. In the present study the muscular layer of the cow and the pig showed MAO activity when kynuramine was used as a substrate or when the histochemical method of Glenner *et al.* (1957) was employed.

The results in the present study are directly comparable to the same author's earlier 5-HT studies (Penttilä 1966), because the specimens were taken in the same anatomical sites of the different species. There was no systematic correlation between MAO activity and 5-HT stores. The sheep had the greatest 5-HT content in the duodenum among the species investigated, whereas its MAO activity was the lowest. In the mouse the reverse applied, and in the guinea pig both the MAO activity and the 5-HT content were remarkably high as compared with those in the other species.

Using Falck's (1962) modification of the fluorescence method due to formaldehyde (Eränkö 1955) it was possible to locate 5-HT almost selectively in the enterochromaffin system of the duodenum (Penttilä 1966). The main location of enterochromaffin cells in all the species investigated was the basal part of the

mucous membrane in the crypts of Lieberkühn. In contrast to 5-HT MAO activity was weak or almost negative in that part of the mucous membrane where the enterochromaffin cell density was at its highest. However because EC are scattered also among the surface epithelial cells, at least in this part of the mucosa they must contain MAO activity owing to the uniform and strong histochemical staining of the epithelial sheet.

The non systematic correlation between MAO activity and 5-HT stores stated in the present study agree with the observations by Tissari (1966) in the developing guinea pig duodenum and by Penttilä and Mustakallio (1967) in the chicken duodenum during pre and postnatal development. Also the observations made by Klingman *et al.* (1964) in the adult rat s gastrointestinal tract agree with the present results, and they further found that dopa-decarboxylase and norepinephrine had no correlation to each other Similar observations were made by Håkanson *et al.* (1966) in the pineal gland whereas Bogdanski and Udenfriend (1956) reported that 5-HT and MAO have a parallel distribution in the dog and the cat brain.

It is probable that 5-HT is protected for MAO activity in the enterochromaffin granules and that this granule amine determines practically the duodenal 5-HT content. It is assumed that 5-HT is produced in the gastrointestinal enterochromaffin system and released from there into the blood circulation (Erspamer 1961) It seems therefore natural to assume that in the mucous membrane where 5-HT is high MAO activity would be weak or negative and the release of 5-HT into the blood circulation possible occurs. On the other hand, 5-HT applied in a very small centration on the mucous membrane causes a vigorous intestinal motility (Bülbring and Lin 1958) and thus, at least the luminal 5-HT participitates in the regulation of the intestinal motility The enterochromaffin cells in the different parts of the mucous membrane may have different functions the basal cells may liberate 5-HT into the blood circulation, whereas those in the epithelial sheet perhaps excrete it into the intestinal lumen. This dual function is supported by the histochemical MAO localization and by the fluorescence studies (Penttilä 1966) in which 5-HT was especially often seen to reach the free intestinal lumen in the epithelial enterochromaffin cell cytoplasm.

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Uptake of Noradrenaline in the Isolated Perfused Rat Heart after Depletion with Decaborane

By

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Abstract

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After depletion of noradrenaline (NA) *in vivo* with decaborane, isolated perfused rat hearts rapidly take up NA to approximately the normal content from a perfusion medium containing NA 10^{-} —10^{-6} M. The NA uptake is inhibited to varying degrees by cocaine, desmethylimipramine phenoxybenzamine, reserpine, iso-methyl nicotinium bromide prenylamine and by certain sympathomimetic and other autonomic drugs. The decaborane-depleted heart appears be ... eful preparation for the study of the bulk uptake of NA.

It is now generally agreed upon that in physiological conditions the uptake of released or exogenous noradrenaline (NA) is effected both through a reuptake mechanism in the adrenergic axon terminals (MacMillan 1959 Whitby Hertting and Axelrod 1960 Muscholl 1961) and through uptake in storage granules (Euler and Lishajko 1965b 1967) This uptake phenomenon is influenced by various drugs including the congeners of neurotransmitters.

Several workers have studied the uptake mechanisms *in vitro* and *in vivo* (Axelrod, Weil-Malherbe and Tomchick 1959 Strömblad and Nickerson 1961 Wegmann and Kako 1961 Muscholl 1961 Iversen 1963 Stjärne 1964 Burgen and Iversen 1965 Euler and Lishajko 1967) Most of the previous studies, however have been performed either by measuring the increase in the catecholamine (CA) content of organs above the normal level after injection or infusion of a relatively large amount of the amine or by studying the uptake by using a tracer dose of radioactively labelled amines. Apparently in such studies it is difficult to differentiate between net accumulation at various storage sites and simple exchange of amines.

Abbreviations used NA (noradrenaline CA (catecholamine) DMI (desmethylimipramine) PBA (phenoxybenzamine) PEA (phenethylamine) A (adrenaline) TA (tyramine)

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Merritt, Schultz and Wykes (1964) first reported that the boron hydride decaborane ($B_{10}H_{14}$) caused a decrease of the CA content in the rat brain. Subsequently Euler and Lishajko (1965a) have shown that in the rabbit this agent gradually depletes the CA stores in various organs, that the depleted heart can take up injected NA to nearly normal values, and that the NA taken up is distributed in the normal fashion.

Later studies have indicated that the depleting effect is due to inhibition of dopa-decarboxylase action (Merritt and Schultz 1966).

The present study deals with the NA uptake in the isolated perfused rat heart depleted with decaborane when infused with NA at different concentrations and with the influence of varying concentrations of sympathomimetic amines and other agents on such uptake. The use of depleted organs seems to afford special advantages for the study of the mechanism of physiologically demonstrable uptake, in difference to the micro-uptake or exchange studied with tracer amounts or the temporary bulk uptake above the normal transmitter level, observed with large doses.

Methods

Female Sprague-Dawley rats weighing 200–250 g were used. Decaborane was dissolved in minimum amount of ethanol, diluted with phosphate buffer (pH 7.4) and injected i.p. in 10, 15 and 20 mg/kg doses, and allowed to act for different time periods in order to ascertain the optimum dose and time needed for depletion. In the uptake studies, doses of 15 mg/kg were given 24 hrs before the perfusion, since this treatment proved to give constant and suitable depletion. Some rats were treated in addition to decaborane with reserpine (1 mg/kg i.p.), desmethylimipramine (DMI) (10 mg/kg i.p.) and phenoxybenzamine (PBA) (10 mg/kg i.p.) 2 1/2 and 1 h respectively before perfusion.

The rats were killed by a blow on the head and the hearts were removed immediately and perfused by the Langendorff technique with Krebs-Ringer bicarbonate solution. The composition of the solution was (in g/l): NaCl 6.9, KCl 0.35, $CaCl_2$ 0.277, $MgCl_2 \cdot 6H_2O$ 1.24, KH_2PO_4 0.16, $NaHCO_3$ 2.1 and glucose 1. The perfusion was carried out at constant pressure and a rate of 6±1 ml/min. The temperature of the perfusing solution was 38° C. The solution was oxygenated with 95 % O_2 and 5 % CO_2 which gave a pH value of about 7.4—7.5. A strain-gauge transducer was attached to the apex of the heart through a pulley in order to record the amplitude and rate of contraction.

After a 10 min control perfusion period, NA at different concentrations was infused for 10 min through a side tube, the tip of which was laid in the perfusion cannula. A further 10 min period of perfusion with Krebs-Ringer solution followed in order to wash out the extracellular NA. According to Lemon (1963) even a shorter washout time should be sufficient for this purpose. In uptake studies, the pharmacological agents were infused simultaneously with the NA solution, while propranolol and in some experiments PBA, were infused during a 10 min period before the NA infusion. All solutions were made fresh in the perfusion fluid. NA solutions contained ascorbic acid in about 100 µg/ml.

After the washout perfusion, the hearts were removed from the perfusion apparatus, freed from extraneous tissues, blotted on filter paper and weighed. They were homogenized and extracted with 10 % trichloroacetic acid in an Ultra Turrax apparatus (Janke & Kunkel KG). The extracts were subsequently adsorbed on alumina, eluted with 0.25 N acetic acid and estimated fluorimetrically according to the method of Euler and Lishajko (1961).

The drugs used in the present study were: l-NA bitartrate, tyramine (TA) hydrochloride, phenethylamine (PEA) hydrochloride, ephedrine hydrochloride, propadrine hydrochloride, metaraminol (Aramine®) bitartrate, prenylamine gluconate (Segontin®), nialamide, phenoxybenzamine (PBA) hydrochloride, desmethylimipramine (DMI), propranolol hydrochloride and N-methyl nicotinium bromide (MN). Except MN which was used as the hydrobromide salt, and PBA hydrochloride, all the concentrations refer to the bases.

TABLE I Effect of decaborane on NA content of rat heart

Group	Number of rats	Dose mg/kg	Time hrs	NA content $\mu g/g \pm$ S.E.	of normal $\pm$ S.E.
Control	7	—	—	0.74 ± 0.04	100 ± 5.83
Decaborane	3	10	4	0.70 ± 0.05	45 ± 2.8
	3	10	12	0.38 ± 0.04	51 ± 3.1
*	3	15	12	0.28 ± 0.03	38 ± 3.8
	8	15	24	0.24 ± 0.05	32 ± 2.65
	6	15	48	0.22 ± 0.03	30 ± 3.4
	2	20	12	0.30 ± 0.02	41 ± 2.6

Not significant (p 0.05) when compared with control group

The effect of the drugs is expressed as per cent inhibition of the NA uptake. The uptake is represented by the difference between the NA content of the previously depleted heart after perfusion with 10^{-7} M or 10^{-6} M NA (0.62 ± 0.03 $\mu g/g$ and 0.83 ± 0.06 $\mu g/g$ respectively, M ± S.E.M.) and the basic value after depletion (0.24 ± 0.03 $\mu g/g$). The NA content of the untreated heart was 0.74 ± 0.04 $\mu g/g$) (cf. Table I and II)

Results

Depletion of cardiac NA with decaborane

Table I summarizes the effects of decaborane treatment with different doses acting different time periods on the NA content of the heart. A standard dose of 15 mg/kg i.p. was used, giving a mean NA content after 24 hrs of 0.24 ± 0.03 $\mu g/g$ corresponding to 68 per cent depletion.

The dose of 15 mg/kg i.p. produced visible muscular weakness within 30 min and sedation or hypnosis with lack of spontaneous movement within 3—4 hrs when the NA content of the heart was still largely unaltered. In many cases the rats lost the righting reflex after 4 hrs. After 20—24 hrs the palpebral fissures were still narrow or at times completely closed. Hyperexcitability with clonic convulsions, sometimes followed by death, was observed in some cases.

Uptake of NA by the isolated depleted hearts

The hearts from the decaborane pretreated rats were perfused for 10 min with Krebs-Ringer solution followed by perfusion with one of the three different concentrations of NA, 10^{-7} M, 10^{-6} M or 10^{-5} M. The results show that infusion of 10^{-7} M NA for 10 min filled up the heart to about 84 % of its normal value of NA or from 0.24 ± 0.03 $\mu g/g$ to 0.62 ± 0.03 $\mu g/g$. The net uptake 0.38 $\mu g/g$, thus represents about one half of the normal value. With NA concentrations 10^{-6} M and 10^{-5} M the hearts were filled up to about 122 and 176 per cent respectively (Table II). Thus the depleted hearts show a remarkable ability of NA uptake although some of this may be extra-neuronal particularly with the higher NA concentrations in the perfusion fluid. In the perfusion experiments of Lindmar and Muscholl (1964) and

TABLE II Uptake of NA by the decaborane pretreated (15 mg/kg i p 24 hrs before) rat hearts when infused with different concentrations of NA

Infusion	Number of rats	Conc. of NA M	Heart NA µg/g ± S.E.	% of infused NA taken up ± S.E.	% of normal NA content ± S.E.	refilled ± S.E.
Control	8	–	0.24 ± 0.028	—	32 ± 2.7	—
NA	8	10^{-7}	0.62 ± 0.031	39 ± 4.7	84 ± 5.6	80 ± 7.3
NA	4	10^{-6}	0.85 ± 0.057	5.6 ± 1 4	109 ± 7.3	123 ± 12
NA	3	10^{-5}	1 12 ± 0.13	0.85 ± 2.9	151 ± 16	176 ± 20
Decaborane + Reserpine						
NA	3	1	0 18 ± 0.02	—	24 ± 1 4	—
NA + nialamide	2	1	0.21 ± 0.014	—	28 ± 2 4	—

Iversen (1963) substantial uptake was observed in nondepleted rat hearts when NA was infused in concentrations of 10^{-7}—10^{-5} M. Fig 1 shows th upake of NA in the depleted heart using the three concentrations of NA in the perfusion fluid.

As seen in Fig 1 reserpine (1 mg/kg i.p.) given 3—4 hrs before infusion, prevents uptake of NA nfused at concentration of 10^{-6} M. Neither was any uptak of NA observed n reserpine-pretreated rat hearts if nialamide 10 M was added to the perfusion fluid.

In some experiments the heart rate vas recorded during the NA uptake tudies as shown in Fig. 2. The heart rate increases sharply with the start of the perfusion and levels down within about 2 min of infusion. This is seen both for the initial perfusion with NA and the subsequent perfusion with TA.

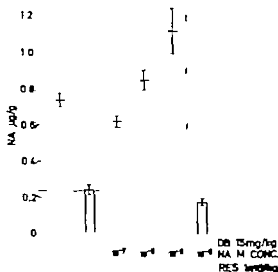


Fig. 1 Uptake of NA in decaborane pretreated isolated perfused rat hearts Decaborane (DB) 15 mg/kg i p was given 24 hrs before. Columns 3—5 NA content (mean ± S.E.) after perfusion for 10 min of 10^{-7} M, 10^{-6} M and 10^{-5} M NA Column 6 NA content after perfusion of 10^{-6} M NA in hearts from rats treated with decaborane + reserpine (1 mg/kg i.p 2 hrs before) Ordinate NA µg/g heart.

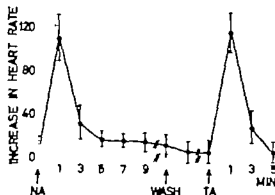


Fig. 4. Effect of NA perfusion 10⁻⁸M on frequency of isolated perfused decaborane-pretreated rat hearts. The subsequent perfusion of TA is shown after wash-out period. Each point represents the mean ± S.E.M. of 5 expts.

Effect of sympathomimetic amines and other agents on NA uptake in decaborane pretreated rat hearts

Groups of 3 rats, treated with decaborane (15 mg/kg i.p. 4 hrs before) were used for each concentration of NA and the antagonists. The drugs were dissolved in different concentrations in the infusing solution together with NA. DMI was administered to the decaborane pretreated rats about 30 min before starting the perfusion, while PBA and propranolol were infused for 10 min prior to NA infusions. In Table III the effects of all the compounds tested have been presented

Effect of PEA and TA

PEA and TA in a concentration of 1.4×10 M completely prevented the uptake of NA when the heart was perfused with NA 10^{-7} M. When NA was present in 10 M concentration the inhibition was less complete but the uptake was still reduced by 71 and 43 per cent respectively. Increasing the drug concentration to 1.4×10 M again increased the degree of inhibition of NA uptake from a 10^{-6} M solution. The effect therefore is dependent on the relative concentrations of the NA and the drugs, indicating competitive inhibition (Fig. 5)

Effect of l-ephedrine, propadrine and metaraminol

All of these three compounds are β-hydroxylated and α-methylated and metaraminol, in addition, has a phenolic hydroxy group. As seen from Table III metaraminol (10^{-} M) completely inhibited the NA (10^{-8} M) uptake while at the same concentration levels ephedrine and propadrine inhibited about 37 and 82 per cent respectively. In order to obtain the same degree of inhibition of uptake at 10^{-7} M NA perfusion the concentration of ephedrine had to be about 10 times higher than that of propadrine

Effect of iso-methyl nicotinium bromide (IMN) and prenylamine

IMN (0.65×10^{-4} M) did not have any pronounced inhibitory effect on the uptake in the depleted rat hearts with NA 10 M but inhibited the uptake of NA 10 M

TABLE III Influence of varying concentrations of sympathomimetic amines and other agents on the uptake of NA by the isolated perfused decaborane-pretreated rat hearts when infused with two different concentrations of NA. Control level of NA 0.74 ± 0.038 µg/g. Control level after depletion 0.24 ± 0.028 µg/g

Drug	Conc. (M) of drug in perfusion fluid	Conc. (M) of NA in the perfusion fluid	Heart NA content µg/g ± S.E.	Per cent Inhibition ± S.E.
PEA	1.4×10^{-6}	10^{-6}	0.42 ± 0.029	71 ± 5.2
	1.4×10^{-5}	10^{-6}	0.13 ± 0.004	100
	1.4×10^{-4}	10^{-6}	0.11 ± 0.004	100
TA	1.4×10^{-6}	10^{-6}	0.59 ± 0.03	43 ± 2.8
	1.4×10^{-5}	10^{-6}	0.49 ± 0.066	60 ± 2.6
	1.4×10^{-4}	10^{-6}	0.23 ± 0.025	100
l-ephedrine	10^{-6}	10^{-6}	0.62 ± 0.024	37 ± 3.9
	10^{-5}	10^{-6}	0.49 ± 0.007	60 ± 0.38
	10^{-5}	10^{-7}	0.37 ± 0.025	66 ± 2.7
	10^{-4}	10^{-7}	0.26 ± 0.028	95 ± 3.2
Propadrine	0.9×10^{-5}	10^{-6}	0.33 ± 0.029	82 ± 3.2
	0.9×10^{-4}	10^{-6}	0.23 ± 0.008	100
	0.9×10^{-5}	10^{-7}	0.27 ± 0.015	82 ± 1.8
Metaraminol	10^{-4}	10^{-6}	0.18 ± 0.012	100
IMN	0.65×10^{-5}	10^{-6}	0.64 ± 0.051	35 ± 5.2
	0.65×10^{-6}	10^{-7}	0.60 ± 0.019	7.4 ± 4.8
	0.65×10^{-5}	10^{-7}	0.28 ± 0.013	89 ± 4.9
Prenylamine	0.5×10^{-5}	10^{-6}	0.52 ± 0.011	47 ± 1.4
	0.5×10^{-5}	10^{-7}	0.28 ± 0.04	90 ± 0.63
Cocaine	0.33×10^{-6}	10^{-6}	0.63 ± 0.035	31 ± 5.0
DMI	Pretreated	10^{-6}	0.23 ± 0.011	100
PBA	0.55×10^{-6}	10^{-6}	0.54 ± 0.026	49 ± 4.2
	0.55×10^{-6}	10^{-7}	0.41 ± 0.02	53 ± 1.8
	0.27×10^{-4}	10^{-6}	0.26 ± 0.02	96 ± 2.1
	Pretreated	10^{-6}	0.21 ± 0.046	100
Propranolol	0.57×10^{-6}	10^{-6}	0.63 ± 0.012	36 ± 2.0
	1.4×10^{-6}	10^{-6}	0.50 ± 0.034	57 ± 3.1
	0.57×10^{-6}	10^{-7}	0.38 ± 0.007	62 ± 3.2

p to about 90 per cent. Prenylamine (N 3-phenylpropyl (2) 1 1-diphenylpropyl (3 -amine) (Segontin®) in the concentration of 0.5×10^{-5} M reduced the uptak f NA (10^{-6} M) by 47 per cent, while in the lower concentration of NA (10^{-7} M) th hibitory action increased to 90 per cent. Both the nicotine analog and prenylamine ha e NA-depleting action (Schone and Lindner 1962, Euler and Haglid to be published) These compounds, as the present study shows, also inhibit th NA uptake (Fig 4)

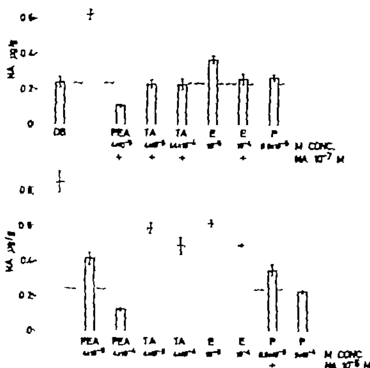


Fig. 3 Uptake of NA in decaborane-depleted rat hearts on simultaneous perfusion with NA and other sympathomimetic amines in different conc. Upper panel: NA conc. 10 M. Lower panel B: NA conc. 10^{-6} M. DB = decaborane. PEA = phenethylamine, TA = tyramine, E = ephedrine, P = propadrine; NA µg/g heart.

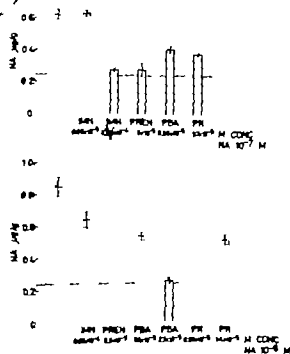


Fig. 4 Uptake of NA in decaborane-depleted rat hearts when different conc. of NA and drugs were used. PBA and propranolol were infused for 10 min before NA infusion. Upper panel: 10 M NA conc. Lower panel: 10 M NA conc. IMN = isomethylnicotinic acid hydrazide, PREN = prenylamine, PBA = phenoxybenzamine, PR = propranolol.

Effect of cocain DMI PBA and propranolol

After DMI pretreatment no uptake was observed with NA 10^{-6} M, while cocaine (0.55×10^{-4} M) produced only about 50 per cent inhibition, and PBA in the same concentration 50 per cent inhibition of uptake. Again, the inhibition was somewhat greater with the weaker NA concentration (10^{-7} M). Pretreatment of rats (10 mg/kg i.p.) with PBA 1—2 hrs before infusion of NA (10^{-6} M) produced complete inhibition of uptake. Infusion of PBA (0 $7 \times 10^{-}$ M) for 10 min followed by NA (10^{-6} M) also produced almost complete blockade. In the experiments of pretreatment plus simultaneous addition, the hearts became, after about 4 min of NA infusion, very feeble and the perfusion rate was very much reduced.

Since the receptors in the cardiac muscle are considered to be of the β-type the effect of propranolol was tested on decaborane pretreated isolated heart preparations. At 0.58×10^{-6} M propranolol 36 per cent of the NA uptake was inhibited when the NA concentration was 10^{-6} M, while 1.4×10 M propranolol, almost the maximum the heart could tolerate, inhibited 57 per cent. Propranolol was about equally active as PBA when the NA concentration was 10^{-7} M.

Discussion

The present study shows that in the decaborane-depleted perfused heart, perfusion with low concentrations of NA, 10^{-7} M and 10^{-6} M efficiently fills up the stores. It is of interest to note that about 40 per cent of the infused NA in the lower concentration was extracted by the heart. With higher concentrations of NA in the perfusion fluid the NA level in the heart even exceeded the normal value. Since a 10 min washout period was always given it follows that the stores of the sympathetic nerve endings not only efficiently concentrate the neurotransmitter but also can retain it. Euler and Lishajko (1965a) have demonstrated repletion of the neurotransmitter stores of the rabbit heart after decaborane followed by slow depletion. Mackenna (1965) has shown that prenylamine-depleted rabbit hearts are capable to take up and store exogenous NA. In this respect, reserpine action differs from that of decaborane and prenylamine, since in reserpine-pretreated rats the restitution of the neurotransmitter stores is in general small and of short duration. The injected NA then mostly appears in the soluble or supernatant fraction, suggesting a longlasting impairment in the retention and accumulation mechanisms in the granules (Euler and Lishajko 1965b Bhagat, Bhattacharya and Dhalla 1966). As seen in the present experiments, the uptake and storing ability was abolished when decaborane-depleted rats were treated with reserpine about 2 hrs before the NA infusion.

Using bovine splenic nerve granules, Euler and Lishajko (1965b) observed that prenylamine in a concentration of $3 \times 10^{-}$ M, like reserpine, wholly inhibited the uptake of NA. In the present studies, when the prenylamine concentration was 5×10^{-6} M and that of NA 10^{-6} M and 10^{-} M in the perfusion fluid, there were about 50 and 90 per cent inhibition of uptake respectively.

The potentiation of the action of exogenous CA by TA, amphetamine ephedrine and metaraminol has been described (Bhagat, Bhattacharya and Wong 1965, Bhagat *et al* 1966 Trendelenburg 1966) Most of the sympathomimetic amines and many of their analogues are quite efficient in inhibiting the NA uptake (Burgen and Iversen 1965) In the present studies all of the indirectly acting amines were potent uptake inhibitors and their effectiveness was more pronounced in the higher concentrations and also more marked at lower perfusion concentrations of NA. Among the β-hydroxylated compounds—metaraminol, ephedrine and propadrine (norephedrine)—metaraminol was the most potent while propadrine was more effective than ephedrine This is in accordance with Iversen's observation that N substitution decreased the affinity of the drug for the uptake site Phenethylamine was about as potent an inhibitor as propadrine. IMN (iso-methyl nicotinium bromide) which inhibits the uptake of radioactive NA in the isolated perfused guinea pig heart (Hedqvist to be published) also exerts a moderate inhibitory action on the bulk uptake of NA in rat hearts.

Cocaine PBA and dichloroisoproterenol (DCI) have been reported to promote the outflow of NA on stimulation of the sympathetic nerve supply to the tissues and potentiate the action of the neurotransmitter (Brown 1965 Muscholl 1961) In the present sets of experiments, cocaine produced only 30 per cent inhibition of uptake when the NA concentration used was 10^{-8} M. Iversen (1963) also observed that the inhibition of NA uptake by cocaine was reduced when the concentration of NA was increased Furchgott *et al* (1963) have suggested a competition between cocaine and NA for a common uptake site

In the present studies, DMI PBA and propranolol were all effective in inhibiting NA uptake DMI pretreatment thus completely inhibited the uptake while the β-blocker propranolol ($1.4 \times 10^{[illegible]}$ M) produced 50 per cent inhibition. Lindmar and Muscholl (1964) have shown that the α blocker PBA and the β-blockers DCI and pronethalol inhibit the uptake of NA (*cf* Euler and Lishajko 1966) In prenylamine-depleted rabbit Mackenna (1965) observed only a small or no blocking effect of DCI while PBA had a pronounced effect. In the present study PBA almost completely blocked the uptake when injected 1 hr before perfusion when infused for 10 min before NA infusion, its effectiveness was lower and comparable to that of pronethalol Th[illegible] it appears that PBA needs time for attaching itself to its site of action, the nature of which [illegible] still unknown. Moreover with PBA and propranolol the effectiveness in inhibiting the uptake did not differ much with the two concentrations of NA

Recently Titus *et al* (1966) inferred that DMI acts at a site in the axon membrane which is probably [illegible] of the system for the uptake of NA and which has some affinity for a [illegible] amines In many cases an inhibitory action of drugs has been demonstrated at the [illegible] level. In the case of some other drugs, however inhibition of the granular NA uptake seems to be lacking in moderate concentrations (*e.g* cocaine [illegible] bromide) suggesting that the inhibition of NA uptake in the heart is mainly at the axon membrane

Even if the decaborane-depleted heart does not allow discrimination between actions on uptake at the axon membrane and in the granules it provides a convenient system for studying the effect of drugs on the bulk NA uptake within physiological limits. The uptake of tracer amounts of NA in a heart with normal amounts of endogenous transmitter does not necessarily follow the same course.

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Cardiac Rhythm during Breath Holding and Water Immersion in Man

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Abstract

Paulev P *Cardiac rhythm d ring b eath holding and water mme sion in man*
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A report is given of the pulse alterations in 30 subjects during face immersion with and without apnea. The breath was held after one maximal inspiration, t transmural, thoracic pressure of zero to +5 mm Hg Apnea in itself produced decrease in pulse averaging 9 per cent of the control pulse (mean. 75 ± S.E. 2.2 beats/min) Apnea plus immersion of the face i 15 C water produced 14 per cent fall, and face immersion in 15 C water without apnea produced 7 per cent fall.—I 11 supine subjects the pulse response during apnea was found to be function of the transmural, thoracic pressure, with little difference between the response in air and in 36 C water.—Both the bradycardia provoked by apnea plus face immersion and that provoked by apnea during whole body immersion is temperature dependent. It is concluded that the bradycardia observed during apneic diving in man, originates from tw independent factors. One of these is an apnea bradycardia elicited from the intrathoracic, high pressure baroreceptors, and the other is bradycardia elicited by cold stimulation of the skin. Thus, the alidity of Irvings concept of a general ertebrate diving reflex (with unknown receptors) common to animals and man, seems dubious.

In the past the bradycardia occurring at pneic diving have been considered as caused by similar mechanism in animals and man (*f* Irving 1964 Elsner and Scholander 1965 Andersen 1966) Although this unitary theory has ppealed to many (Scholander 1962 Olsen *et l.* 1962, Irving 1964 Sasamotu 1965 Wolf 1965, Andersen 1966, Brick 1966, and Corriol *et al.* 1966) the facts that the diving pat terns of diving animals and of man differ considerably and that the reported brady cardias are often of different magnitudes, may indicate that w are dealing w th bradycardia phenomena of different nature. Most diving animals exhale prior to submersion and show a ery pronounced bradycardia (Eliassen 1960 Murdaugh *et al.* 1961 Feigle *et al.* 1963 Reite *et al.* 1963, and Andersen 1966) in seals for example the heart rate falls to one tenth of the normal heart rate in the emerged state (Irving 1964) Human breath-hold divers inhale prior to submersion and the bradycardia is ery small. The bradycardia found in different diving animals is not necessarily a physiological entity with regard to the eliciting mechanism, and even

in the best studied animal the duck, the nature of the involved receptors is unknown (Andersen 1966) Therefore it seems premature to extend the names "diving bradycardia" or "the oxygen conserving reflex" arising from studies of diving animals, to the bradycardia observed during human apnoic diving

The present study was performed to investigate the nature of the bradycardia reported to occur in human subjects during breath-hold diving. Preliminary studies showed that the size of the heart rate response upon apnea is governed mainly by the size of the transmural pressure across the thorax (lungs plus chest wall) and by the environmental temperature. Since both the face immersion bradycardia of man the bradycardia described among human breath-hold divers, in previous studies was considered of a similar origin as the "diving bradycardia" of animals (Elsner *et al.* 1965 Wolf 1965 Andersen 1966 Brick 1966 and Corriol *et al.* 1966) two experimental series were performed In the first of these the heart rate was measured in the prone position during apnea with and without the face immersed in water at different temperatures. In the other series the heart rate was measured during apnea while the subject rested in water to the neck level for 30 min.

Methods

In this paper "apnea" is used synonymously with breath-holding The transmural pressure across the thorax is defined as the airway (mouth) pressure minus the external pressure In air the external pressure is the atmospheric pressure P_B, whereas when lying horizontal in water it is assumed to be the hydrostatic pressure at the mid axillary line

The 30 subjects used were healthy medical students and doctors aged between 21 and 3[illegible] yrs. Seven of these were accomplished swimmers (i.e. swim in a swimming pool or in the [illegible] at least once a week all year round) All breath-holds of this study were performed after maximal inspiration (see below) and no hyperventilation was permitted preceding the apnea. The breath-holding time [illegible] the duration of the other manoeuvres, was always 60 sec [illegible] After a single maximal inspiration —without previous overbreathing—all subjects were able to maintain the apnea for 60 sec without involuntary contractions of the diaphragm, as judged by the constant airway pressure Between periods of apnea, respiratory excursions were followed by means of a pneumograph around the chest. During breath-holding the airway pressure (at the mouth) was measured with a water manometer (Knowles *et al.* 1959) The heart rate was recorded [illegible] th [illegible] electrodes connected to a sensitive mirror galvanometer by shielded single conductor cable All parameters were recorded with an ultraviolet light recorder the pressures via Statham transducers Temperatures were measured with thermistors in the rectum and on the skin (Crane and Dvorak 1966) Separate from the main experiments the size of the vital capacity (VC) and the expiratory reserve volume (ERV) was measured with a low resistance spirometer Also the last inspiration from ERV prior to an apnea was measured repeatedly in a separate experiment Although the subjects claimed that they initiated the apnea after maximal inspiration measurements showed volumes ranging from 84 to 100 per cent of VC when the subjects inspired from the functional residual capacity level (FRC) before each apnea. Although the inspired lung volume in each individual was reproduced with great constancy [illegible] likely that the subjects did not achieve total lung capacity (TLC) prior to apnea, but for the sake of simplicity the initial apnea lung volume (mean 90 per cent of VC) in the following [illegible] ed as TLC

The "heart rate" is here used [illegible] the total number of heart beats (all R R distances counted) in 60 sec periods. E[illegible] en d[illegible] ring [illegible] ad [illegible] counting of the number of heart beats for periods less than 20 sec showed an [illegible] lack of reproducibility indicating an advantage in using all heart beats in 60 sec periods as representative of the "heart rate" The heart rate response to a specific manoeuvre was expressed as the percentage change during the 60 sec manoeuvre, from that of the 60 sec [illegible] (control) period preceding it. If not otherwise indicated 30 observations were performed [illegible] each group of experiments on the 30 subjects The mean percentage change ± the standard error (S E) is shown throughout, except in Fig. [illegible] where the standard deviation (S D) is indicated.

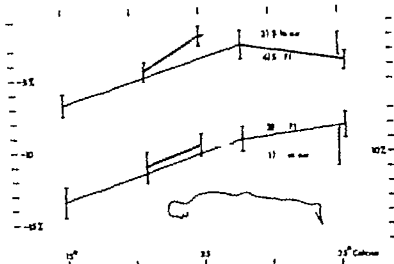


Fig. 1. Abscissa: water or air temperature. Ordinate: percentage decreases of the resting control heart rate at the different manoeuvres. 1) Apnea in air (room air and "face ventilation"). 2) Apnea plus face immersion in water. 3) "Snorkel" breathing in air. 4) "Snorkel" breathing plus face immersion in water.

At each point is shown the mean value of 50 observations (from 50 subjects) except at apnea in warm air (30 observations only) and apnea in room air (97 observations). The vertical bars indicate S. E. M. Average resting (control) heart rate 75 beats per min.

Two groups of experiments were performed: I) Face immersion in the prone position, and II) whole body immersion in the supine position. In the literature both procedures are considered able to elicit the so-called "diving bradycardia" of man (Craig 1963, Andersen 1963).

I. *Prone position (face immersion and controls)*

The subject was hung prone on a table with the head and neck extending beyond the border, the weight of the head being carried by a head sling covering the eyes (Fig. 1). A noise level of 80 db from a …ulator was used during the whole experimental period to mask disturbing sounds. The nose was closed by a nose clip. During apnea the subject kept a transmural, thoracic pressure of zero to +5 mm Hg. The following manoeuvres were performed: 1) apnea plus "face ventilation" (see below), 2) apnea plus face immersion, 3) "face ventilation" without apnea, 4) face immersion without apnea, 5) forearm immersion without apnea, 6) mental stress.

In 1 and 3 air passed the subjects face during the manoeuvres ("face ventilation"). The temperature of the air blowing around the face was 35 or 21 C. The manoeuvres were also performed in general room air (25 C) without "face ventilation".

In 2 and 4 the face immersion was performed by raising a water basin sufficiently for the level of water (35.5, 28° and 15 C) to rise to 3 cm below the subject's ears.

In 3 and 4 the subject breathed through a respiration valve with two 50 cm rubber tubes for 3 min (two-way "snorkel" breathing): 1 min precontrol, 1 min "face ventilation" or face immersion, and 1 min postcontrol.

In 5 the hand and lower part of the forearm were immersed in water (35.5 and 15 C), the skin area are being approximately the same as in the face immersion procedure.

In 6: To examine the influence of intellectual emotional activity during such procedures, the subject was given 60 sec to raise three to the power of seven with mental arithmetic.

The order of the experiments was varied so that the same order of sequence was not followed twice. An assistant started each of the above manoeuvres by counting aloud the last 5 sec down to the time zero. The subject was instructed to start his maximal inspiration 1 sec before zero. Each time another assistant inserted a mouth piece connected to the water manometer.

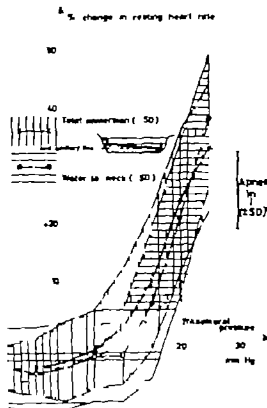


Fig. 2. The mean heart rate response (±S.D.) to breath-holding as a function of the transmural, thoracic pressure. The water temperature was 35.5 to 36 C, and the room temperature was 23 C. The curves are the mean values of 1 observations in 6 subjects. Mean resting (control) heart rate 7? beats/min.

(b) p positive whole body immersion d co trols)

In order examine the heart rat changes occurring t breath-hold diving, experiments ere made with the subject in a bath tub with circulating, thermostaticly controlled water. Eleven of the 30 subjects were used, one of which was an accomplished swimmer. The first apnea was performed 2 m after the start of the 30 min submersion period, and in the 2 min control periods between the breath-holds the subject rested in water t the neck lev l (Fig 2). T series were made (A nd B). I A the water temperature was kept constant (35.5 C) at an approximate neu al range Craig and Dvorak 1966) and the transmural, thoracic pressure was aried from pnea pnea I B approximat neutral" (zero to +5 mm Hg) transmural, thoracic pressure as kept during pnea, and the water temperature as aried.

A. I 6 subjects three si ations were compared. Breath-holding performed w th (1) water in the bath ub, (2) w t (35.5 C) covering the body p to the mandibular margin, and (3) water (35.5 C) coverin the body and head totally (Fig 2). I these three situations several breath-holds were performed during which the breath was held at transmural, thoracic pressures of − 10 5 ze +5 +10 +20 +25 and +30 mm H th the subject watching the w ter manometer (in (3) by the help of a pai of goggles. Apnea periods with relaxed best wall pproxima 1 +10 mm Hg) nd losed eyes were also performed

B. I the second series each of 5 subjects performed 5 breath-holds in water to the neck level and 5 with total immersion l body and head during the apnea. The experimental order was changed in random fas The first day the water temperature was 35.5 C, whereas the second day it was 22 C \ lescribed abov all bre th-holds were performed at transmural, thoracic pressure of zero 5 mm Hg The cold temperat re 22 C as the lowest temperature tolerable to our nake resting subjects for the 30 min experimental period. The warm temperature is an pproxima neutral range since most physiological parameters are only altered to minimal extent dur 60 min resting stay w te to the neck level (Craig and Dvorak 1966). This holds tru least for tw essential parameters. The control

heart rate did not change significantly and the temperature of a thermistor placed in the rectum did not change during the experimental period, in 35.5 C water. In 22 C water the temperature of the thermistor located in the rectum showed a tendency toward an insignificant increase (0.1 C) after 15 min, but it again approached the initial temperature. The resting (control) heart rate did not change essentially (less than two per cent decrease) during 30 min immersion in 22 C water.

Results

In the below experiments the breath was held at a lung volume of TLC (with the above mentioned reservation). The respiratory depth and frequency was not or only slightly altered, when changing from normal breathing to snorkel breathing. No consistent change in heart rate accompanied these small respiratory alterations.

P one

1) Apnea combined with 35 C air face ventilation elicited a minus 9.7 per cent heart rate response (bradycardia) whereas the combination of apnea with face ventilation using 21 C air gave a response of —11 per cent (Fig. 1). Breath-holding in room air (25 C) elicited a 9.5 per cent decrease of the control heart rate (Fig. 1). In the seven swimmers apnea in room air elicited a response of -7 ± 1.2 per cent.

2) *Apnea plus face immersion.* Apnea combined with immersion of the face in 28 and 35.5 C water yielded almost the same response (—9.2 and —8.6 per cent respectively). Apnea combined with face immersion in 15 C water yielded a heart rate response of —13.4 per cent, and the chance that this value is from the same population group as —9.2 per cent is less than one in thousand ($p < 0.001$). In the 7 accomplished swimmers 11 observations yielded an average heart rate fall of 19 ± 2.8 per cent as compared to 39 observations in the remaining subjects with a mean fall of 12 ± 1.0 per cent. A group of 14 physically fit subjects with a resting (control) heart rate less than 70 (mean 65 ± 1.1 beats/min) showed an average heart rate fall of 11 per cent during apnea plus face immersion in 15 C water. The remaining 16 subjects with a control heart rate greater than 70 beats/min (mean 78 ± 1.0) responded with a fall of 16 ± 1.6 per cent on the same stimulus.

3) *Face ventilation without apnea.* By combining face ventilation and snorkel breathing the heart rate decreased 4 per cent in 21 C air and 2.4 per cent in 35 C air (Fig. 1).

4) *Face immersion without apnea.* Immersion of the face in 28° and 35.5 C water during snorkel breathing yielded almost the same response at the two temperatures (mean —4 and —3.8 per cent, respectively) but in 15 C water a mean response of —6.8 per cent (Fig. 1). The 7 swimmers showed a mean response of -8 ± 1 per cent at 15 C.

5) *Forearm immersion without apnea.* In 22 expts. immersion of the forearm in 35.5 C water elicited a heart rate decrease of 2.4 per cent, while forearm immersion in 15 C water produced a 3.5 per cent decrease (S.E. ± 1 and 0.7 per cent,

TABLE I Heart rat response (percentage decrease of resting heart rat) pon apnea lying supine in water
All breath-holds were performed with transmural, thoracic pressure of zero to +5 mm Hg The results are mean values ±S.E.M. from 5 representati subjects. Fi identical observations were performed on each subject, yielding total number of 25 observations in each of the 4 groups (i.e. 50 bservations t each temperature) The resting (control) heart rat is 71±0.7 beats/min i the warm nd 73±0.8 beats/min in the cold ater

W ter temperature	22.0 C	35.5 C
W ter t the neck	−14±1.5	−8±1 4
Total immersion	−14±1.0	−4±1 7
Sum	−14±0.9	−6±1 0

respectively) when the usual counting down procedure was used. Identical figures were found when the immersion was performed without counting down aloud.

6) *Mental stress* The 14 subjects faced with an arithmetical problem all responded ith a considerable *tachycardia on this — emotional — stress (+17 ±1.0 per cent)*

During the above six maneuvres the resting (control) heart rate of the 30 subjects a eraged 75 beats per minute (±2.2) with the subjects at room temperature dressed and prone The 7 accomplished swimmers also had a mean resting heart rate of 75 but with a S E. of±5 9 beats/min. Thus with a few exceptions, the con l pulse was below 80 beats/min In each subject the control heart rate remained ssentially the same d ring the 30 min procedure.

Supine

A *Results from th 6 subjects performing apnea at different transmural* thoracic pressures are shown in Fig 2. It is seen that the heart rate response with apnea is dependent upon the transmural pressure across the chest wall plus lungs At transmural, thoracic pressures between −5 nd +5 mm Hg there is a slight brady cardia, and at +10 mm the response become one of tachycardia increasing almost linearly with the increase in transmural pressure This apparent dependency of the apnea heart rate on the transm ral thoracic pressure is present, whether the ubject is in air or in water Th air curve have raised with a somewhat smaller slope than the water curves. The diff rence between the results with ater to the neck level and those with total immers on is ery small, and not significant

B. In the 100 expts on 5 ubjects, where the breath was held at transmural thoracic pressures of appro matel zero to +5 mm Hg the apnea was found to cause a decrease of 14 per c nt from the control heart rate when performed by subjects lying in water of 22 C wh le a decrease of 6 per cent was observed in subjects lying in water of 35.5 C (Tabl I) This difference is also highly statistically significant ($p<0.001$) Th results w th water to th neck level and with the total

TABLE II Respiration and heart rat response

	First	Last
Respiratory rate (breaths/min)	19 6±0.7	17.2±0 6
Respiratory depth (cm amplitude)	2.5±0.2	3 9±0 4
Heart rate esponse (per cent hange)	−15.2±1 4	−14 3±1 1

The alues are means and S E.M of 25 observations in each group. The first and the last half part of the immersion period (whole body immersion in 22 C water) is compa ed. The average resting (ontrol) heart rate is 73 beats/min

mmersion during apnea do not differ significantly neither at 35.5 not at 22° C during apnea in per cent of the control heart rate when l ing in water to the neck. The difference between the size of the heart rate response at 22° and at 35.5 C is also evident here In 22 C water the respiration was deeper and less frequent in the last 5 control periods compared to the first 5 periods (Table II) This change in respiration was not accompanied by an change n the control heart rate or the size of the heart rate response to apnea (Table II) In 35 5 C ater the respiratory pattern of the control periods did not hange significantly

In the minute aft   the breath holding (the post apnea cont ol period) the heart rate was 2 per cent greater than in the 60 sec pre apnea control period (the com mon reference alue of this report) t transmural thoracic pressures of −5 mm +5, and +10 mm whereas the heart rat fell to 2 5 per cent below the pr apnea control level in the period follo ing apne at +30 mm Hg (Gorlin t al 1957)

Discussion

Before the general discussion th methods f measurement of the tw ma parameters of the present tud ha   to be considered.

The transmural thoracic pressur w   ec rded during apnea with open glott and with a water manomet   nnected t the mouth of the subject. Our tr i ed s b jects were able to ma ntain the p essu essentially constant durin the 60 se (ne periods both when the   er wat h ng the manometer and when they losed the eyes. Goggles made t poss bl to   tch th manometer even during total mmersion of the head when l ng up ne n w ter Most of the apnea period f t   study were performed t ne l   mplet fill ng f the lungs (90 per ent f VC and with a transmural hor ic pressure f zero to +5 mm Hg Th s   ver l l ... that the subject wer   t ble t rel x their respiratory muscles ... idually they repr d ed he p es   th great constancy from reath-to- breath hold w thout   h ng the manometer These pressures were ... spontaneous by most f he s bjects and they were felt to be the best ... tion to relaxation pres   ... nable   the present ituation. I subject ...

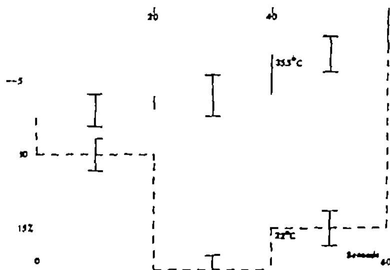


Fig 3. Ordinat percentage decrease of the control heart rate in water t the neck absciss. breath-holding time in seconds Heart rate during apnea counted in 20 sec periods and multiplied w th three The solid curv shows the response upon breath-holding b ng supine in 33.5 C w ter and the dashed curv the response in 22° C water (mean±S E.M. of 30 observations each

perform apnea with an open glottis, the use of the mouth pressure as airway pressure was found a practical and reliable method, as also found by others (Knowles *et al* 1959) The influence of the above variations of the apnea lung volume is not -orta t to the apnea heart rate response (Craig 1965)

The heart rate wa defined as the total number of beats *count d* in 60 sec periods. Fig 3 shows that even by counting in 20 sec periods, one is left with the difficulty to decide which period to chose as representative for comparison. The duration of the counting period chosen by previous workers in the field has varied considerably Measurement of each individual R—R distance before, during and after apnea, was also used prev usl Craig 1963) but due to the rather unsystematic variation of the beat to beat period w th respiration a considerable number of R—R intervals must be combined to defi e a pulse rate level. Previous studies ignored the influence of hyperventilatir nd exercise on the control heart rate prior to apnea Thus by using short control periods with arying and very high control heart rates, substantial—but irreprod ible—pulse responses resulted In the present study the method of counting all heart beats for 60 sec was considered the best a ailable Hyperventilation and mov men t were a oided, and from subject to subject the control (resting) pulse aried between 58 and 85 beats min.

General discussion

So far only Craig has reported on th influence of the intrathoracic pressure on the apnea heart rate response but he disregarded the influence of the hydrostatic pressure

in water and therefore found great differences between the apnea heart rate response in air and in water (Craig 1965) Except for Craig's studies (1963, 1965) other reports often fail to include the information about the apnea lung volume, transmural, thoracic pressure, and the water temperature (Olsen *et al.* 1962, Elsner and Scholander 1965 Sasamoto 1965 and Wolf 1965) A detailed discussion of such reports is not considered pertinent, as the two parameters (transmural, thoracic pressure and temperature) demonstrated in the present study to affect the heart rate during apnea, were either not measured or were not varied with the aim of studying their effect on the heart rate response.

The results of the present study have demonstrated that a significant bradycardia can be accounted for by the apnea *per se* and that the degree of bradycardia elicited by apnea is influenced by temperature, the greatest response being obtained when cold stimuli are added to those created by the apnea. Previous studies of the "face immersion bradycardia of man overlooked the temperature dependency (Brick 1966 Corriol *et al.* 1966, Elsner and Scholander 1965 Irving 1964 Olsen *et al.* 1962, Wolf 1965) The human "face immersion bradycardia has until now been considered as similar to the trigeminal bradycardia, elicited by immersion of the nares of the duck in water (references as above) According to Andersen theirs the bradycardia during apnea in the duck is independent of water temperatures between 15 and 30° C (Andersen 1966) Since the present results show that in humans the bradycardia during apnea is temperature dependent, and of similar magnitude whether the apnea is performed with the face remaining in air or with simultaneous face immersion in water f neutral temperature, a trigeminal reflex with unknown receptors cannot be held responsible.

Thermal factors

P n Apnea in room air created a heart rate decrease of 9.5 per cent (7 beats/min) in the prone position and with a transmural thoracic pressure of +5 mm Hg Essentially the same response is found during apnea with 'face entilation in warm air and during pnea with face immersion in 28 and 35 C water (Fig 1) Thus these reactions may be explained as an apnea response *per se* Apnea with the face immersed in cold w ter evidently adds a further bradycardia to the apnea brady cardia and entilating the face w th cold air (21 C) also seem to add to the apnea bradycardia For technical reasons it was not possible to obtain air temperatures of 15 C, but Fig. 1 inevitably leaves the impression of a temperature dependent heart rate response, just as Table I A possible explanation of the brady cardia responses from the prone maneuvres is a combination of an apnea brady cardia nd a cold bradycardia —Since all maneuvres created bradycardia, whereas the emotional-intellectual tress by calculation yielded a tachycardia, it seems reasonable to assume that th results of the bradycardia maneuvres were not severely influenced by emotional, intellectual activity of the subject during the experiment.

The 7 accomplished swimmers showed a mean heart rate response during breath holding w th the face immersed in 15 C w ter of 19 per cent (15 beats/min) at

a −7 per cent response during apnea in air Thus their apnea bradycardia does not seem to be of a greater order of size than the average value for the whole group (−9.5 per cent) as claimed previously (Corriol *et al.* 1966) and their more pronounced cold bradycardia may simply be due to a smaller emotional intellectual inhibition of the size of the bradycardia, because swimmers are more used to cold water

Supine When resting supine in 35.5 C water to the neck level, apnea elicited a 6 per cent (4 beats/min) decrease whereas in 22° C water apnea produced a 14 per cent (10 beats/min) fall from comparative control heart rates (Table I) This demonstrates that also during whole body immersion the apnea bradycardia can be augmented by a cold stimulus of the skin surface It may be surprising that apnea with face immersion yields a heart rate response of essentially the same size (14 per cent) as during apnea in cold water to the neck. This is explainable because the skin of the face was acutely exposed to cooling simultaneously with the apnea, while in the body immersion experiments (in water of 22° C) the skin of the body had been exposed to cooling for a considerable time before the apnea. Thus adaptative changes are apt to occur to a greater extent in the later case

Mechanical fa tors in apnea bradycardia and diving bradycardia

The relative bradycardia during apnea at low transmural, thoracic pressures and the tachycardia during apnea at high pressures (Fig 2) is explainable by the following mechanism. At transmural pressures of zero mm, the intrathoracic pressure (i e the pressure in the pleural space minus the atmospheric pressure) is approximately 10 mm at TLC (Otis *et al* 1946 and Knowles *et al.* 1959) This intrathoracic pressure is somewhat lower than the pressure with the lungs in the mid position as in the control period and by itself adds a stimulus to the baroreceptors of the aortic arch. Furthermore, by facilitating the venous return to the right heart it should, in accordance with Starl ngs law of the heart (Starling 1918) increase the stroke volume leading—with a short delay—to an increase in systemic arterial pressure This would add a further stimulus to the baroreceptors (aortic and carotid) which in turn could explain the bradycardia elicited by apnea at TLC, with a transmural thoracic pressure f zero (Fig 2) At a transmural thoracic pressure of +8 to +12 mm Hg, no heart rate response pon apnea is to be expected since the intrathoracic pressure of the same—slightly negative value—as in the normal midposition. This was actually found in the 3 experimental series (Fig) On the other hand when apnea is performed at TLC with a tr nsm ral thoracic pressure of +20 to +30 mm Hg the intrathora ic pres re will be positive (+10 to +20 mm) Thus, under such conditions the sit t on will be reversed. there will be an immediate lowering of the stimulus t the aortic receptors followed by a general lowering of the stimulus to the barorec pt rs due to a fall in sy temic arterial pressure resulting from impaired venous return to the heart In fact the expected tachycardia occurred under such conditions (Fig 2) Actual measurements of direct blood pressure during apnea (14 subjects) performed in this laboratory showed right atrial mean

pressures of +1 to +5 mm Hg, and an increase of the mean arterial pressure of 30 per cent from the resting, control values, when apnea was performed at TCL with a transmural, thoracic pressure of zero. Apnea at TLC and a transmural thoracic pressure of +20 to +30 mm yielded an initial fall of 35 to 47 per cent of the resting mean arterial pressure. Thus adequate stimuli to the high pressure baroreceptors are actually present to explain the observed changes in heart rate.

The bradycardia described during apneic diving in man is—according to the present results—interpretable as a combined bradycardia, consisting of two ind pendent bradycardia phenomena. One is an apnea bradycardia of a size dependent upon the transmural, thoracic pressure (Fig. 2) and the other is a cold bradycardia the size of which depends upon the temperature of the water The cold brady cardia demonstrated in man is different from the temperature ndependent diving bradycardia described in ducks. On the basis of these considerations Irvings generalisation—a diving *b adycardia* reflex common to animals and man—is unwarranted. Raper and co-workers found no evidence of oxygen conserving reflexes in man, during apnea in air at an initial apnea lung volume of FRC, and at an assumed airway pressure of zero (Raper *et al.* 1967) Murdaugh *et al* (1961) believed that the bradycardia reported in some vertebrates may reflect nonspecific effects rather than a true diving adaption.

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On the Physiological Significance of the Amplitude of the Endosomatic Galvanic Skin Reaction (GSR) in the Cat

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Abstract

Lang A. H. *On th physiol gi al significanc of th amplitud of the endosomatic gal an sk n action (GSR) in th at* Acta physiol scand. 1968. 73 151—160

Endosomatic galvanic skin reactions (GSR) evoked by electrical stimulation of the distal stump of sectioned sciatic nerv has been ecorded with metal macroelectrodes. It has been established that the amplitude of the negativ phase of the endosomatic GSR is determined by) the stimulation parameters,) the location of the recording electrodes, and (iii) the neuro-sudomotoric activity befor the stimulation I addition, the GSR is influenced by both (iv) spatial summation and ( ) temporal summation or cumulation, and may be influenced ( ) by the area of contact between the lectrodes and the skin. On the basis of earlier and new observ tions in the recording of GSR the onclusion has been drawn tha the negativ phase of the endosomatic GSR recorded with a macroelectrode is compound potential hich is produced in circuit of existin elements and unit potential generators UPG group of effector ells that ar probably situated in the wall of the sweat hannel The UPG's, each of which produce unit potential (UP) seem to be coupled in parallel to each other and in series with the surface electrode

The galv nic skin reactions (GSR) induced in animals, usually cats ha e been studied to some extent from the point of iew of the central regulation of th re action Wang 1964 Bloch 1965) In these studies attention has been mainly directed to differences in the amplitudes of gal anic skin reflexes The q ua t tativ relationsh p between the efferent nerve acti ity and the mpl tud of th or responding GSR has not yet been clarified, however nor is t known h ether factors determine th amplitude of the GSR. The aim of thi stud has been to larif these problems. Only the rapid negative component of he ndosomatic GSR was studied as t probably i the immediate product of the effector lls of the sweat glands and as t is the more dominating of th two component of the GSR in the t La g 1967a

Methods

The methods of inducing and recording GSR s have been described in detail elsewhere (Lang 1967a) Mongrel cats w re employed in the experiments. They were anesthetized with nembutal and urethane and restrained with succinylcholine. Square pulse stimuli, single or repetitive, were applied to the distal stump of a sectioned sciatic nerve. After the nerv was split into two slips, it was possible by success ely and separately stimulating the slips to record two GSR s, if necessary of diff rent intensities. By clamping one stimulated nerv slip (ithout severing the electrical connection) at the end of an experiment, it was possible to liminate one of the GSR s although the stimulation of th clamped slip was continued using supramaximal timulus intensity This could be done without causing any hange in the amplitude of the other GSR. It was thus possible to confirm that the reactions induced by electrical stimulation of the tw nerv slips were not licited by a spreading of the stimulating current to the fibres in the other slip or to the unsplit nerve trunk.

For the recording of the GSR s from central or toe pads of the paw zinc discs 1 cm in diameter or silv rods 2 mm in diameter were employed. The zinc electrodes were coated with an electrode past composed of zinc sulphate, agar and water The surfaces of the silver rods were hlorided by electrolysis and aft this inserted in short piece of rubber tubing which was filled with potassium chloride lectrode paste. The silver electrodes wer ttached to the skin surface with collodion. The latter electrode type was used to record local diff rences in the GSR on the same paw Tw or three lectrodes could be easily applied t the central pad without any short circuits between the lectrodes. I umber of experiments the GSR were recorded with mercury-calomel electrode employing a potassium chloride solution as contact electrolyte. In these experiments the area of contact was aried by immersi g the paw t different depths in the electrolyte solution. The reference electrode was situated on an indifferent skin region, mostly t the tip of the tail.

The GRS were recorded with Grass Model 5 P1 F d.c. preamplifiers and Grass Model 5 six-channel pen-recording polygraph. Because the input resistance of each preamplifier was relatively low (of the order of 200 kohms) the results were checked employing cathode follower and Grass Model P 6 preamplifier N significant differences were established between the results obtained with these two recording apparatus.

The results presented below were obtained in experiments with 25 cats.

Results

1 *The Relation hip betw en the Intensity of the Stimulation and the Amplitude of the GSR* By determining the threshold intensities with square wave pulses of varying duration, typical trength-duration curves could be drawn for the GSR, the chronaxy being of the order of 1 msec. When the intensity of the stimulus exceeded the threshold alue the amplit de of the reaction increased continuously *i.e.* no stepwise ch nges occ rred in the amplitude of the GSR when the intensity of the stimulus was slowly increased The amplitude of the GSR of the central pad evoked by a stimulus of maximal intensity applied to the distal unsplit stump of the sciatic nerve varied considerably from one animal to another Usually the amplitude remained between 10 and 25 mV after a sufficiently long prior activity (see section 6 below and Lang 1967a When the various studied factors influencing the GSR were held constant, some degree of spontaneous variation was noted in the amplitude of the GSR, the relative ariation being greater when low stimulation intensities were employed.

2. *Recording Electrod Loc t a d the Amplitude f the GSR* When the stimulation intensity was held constant the amplitude of the GSR recorded from the central pad was *higher than the amplitudes of the GSR s* recorded from the toe

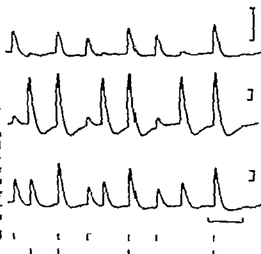


Fig. 1 The fluence of re ording elect ode locatio and ero splitting upon the am plit de of the GRS. GSR's recorded from three different sites on the same central pad. The short vertical lines on the 2 horizontal lines t the bottom of the figure indicate which of the two nerv slips was stimulated and when this took place. Note that both slips were simultaneously stimulated 3 times. The stimulation parameters were 4 V 10 msec (upper line) and 3 V, 10 msec (lower line). Calibrations 2 mV and 10 sec.

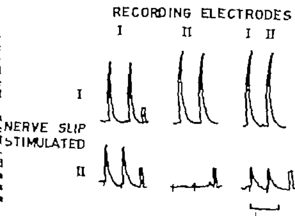


Fig. 2. The depend nc of the amplitude of the GSR th ontact area of th electrodes. The GSR were evoked with two successive identical single impulses applied to the separate sciatic nerv slips, I or II (upper and lower traces respectively). The GSR ere recorded either separately with two electrodes applied to the same central pad (first and second pairs of traces) or with two connected recording electrodes using one amplifier (third pair of traces). The responses in the lower traces are preceded by stimulus artifacts. Calibrations mV (on the traces) and 10 sec.

pads. When the stimulation was applied to a small slip separated from the sciatic nerve local differences in the amplitude of the reaction were observed also on the central pad (Fig 1). In extreme cases the GSR could be recorded from onl part of the central pad (Fig. 2, lower traces). Even a minor local and superficial lesion in the epidermis under a recording electrode was sufficient to reduce the ampl tude of the GSR to a fraction of the amplitude recorded from the same b intact area.

3 *Th Cont ct Area of the Record ng Electrod and th Ampl t d f th GSR* The influence of th contact area of the recording electrod on the amplitude of the GSR is seen in *Fig. 2*. The reactions were recorded from two sites on the central pad with two sil er electrodes whose areas of contact with the kin were approxi mately equal. The distal stump of the sciatic nerve was split into two slips. Stimulation of one of these slips evoked GSR of nearl equal amplitude both recording sites (Fig. 2, the first two upper traces) but when the other slip was timulated

a GSR was recorded with only one of the electrodes (Fig 2, the first two lower traces). When these reactions had been recorded separately with the two active electrodes, the electrode leads were united to double the electrode area. The reference electrode (at the tip of the tail) was the same in all these recordings. The amplitude of the GSR evoked by stimulating the first nerve slip did not differ essentially from the amplitudes of the reactions recorded from a skin area half as small. However the amplitude of the GSR evoked by stimulating the other nerve slip was now only half of the amplitude of the GSR recorded with the other electrode (Fig 2, the third traces on the right).

In the experiments where an electrolyte solution was the contact medium between the electrode and the cat's paw the amplitude of the GSR decreased when the depth of immersion of the paw increased although the stimulation intensity was held constant.

4 The Peak Amplitude of a Fused GSR As has been reported (Lang 1967b) repeated supraliminal stimulation evoked a fused GSR which was characterized by a rapid rise to a peak value and a variable subsequent decrease. Complete fusion was achieved generally already with an impulse interval of 0.5 sec. The peak amplitude of the fused reaction was without exception greater than the amplitude of a phasic GSR evoked by a single stimulus of equal intensity. The phenomenon may be due either to temporal summation or cumulation of reactions.

The peak amplitude of the GSR increased when the stimulation frequency was increased. The maximal peak amplitude was produced with impulse intervals of 0.1—0.03 sec when the stimulation intensity was constant. The weaker the stimulation intensity the larger was the relative increase in the amplitude of the GSR with increasing stimulus frequency. The dependence of this cumulation or temporal summation on the stimulus intensity and frequency is seen in Fig 3. At the lowest

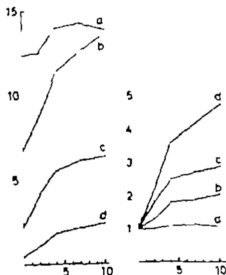


Fig 3 The dependence of the peak amplitude of the GSR on the stimulation frequency and intensity. Abscissa: frequency of stimulation (imp/sec). Ordinate: peak amplitude of the GSR, *absolute values on the left* (mV) and *relative values on the right*. The different curves represent the results from four stimulation series employing different stimulation intensities (a supramaximal intensity 10 msec, b 15 V 10 msec, c 8 V 10 msec, d 6 V 10 msec). The initial values on the ordinate axis on the left diagram are the amplitudes in millivolts of GSR evoked by single impulses of varying intensity. The amplitudes of these same GSR are taken to be unity in the diagram on the right.

stimulation intensity the peak amplitude of the GSR produced by 10 imp/sec was about 5 times as great as the amplitude of the reaction induced by a single stimulation, but when the stimulation intensity was supramaximal the cumulative effect was of the order of 1 1 In this experiment the peak amplitude of the reaction produced by a stimulation frequency of 10 imp/sec was approximately equal to the amplitude of the reaction evoked by a single stimulation with the second highest stimulation intensity

5 *Th Summ tion f the Two GSR s Evoked by Stimulat n of Two Nerve Slips* The GSR evoked by supramaximal impulses (two stimulators) applied successively to two nerve slips of a sciatic nerve generally differed in magnitude. The ratio of the reactions called here partial depended on the locations of the recording electrodes. When the impulses were amplied to both nerve slips simultaneously the amplitude of the resulting combined" reaction was approximately equal to the arithmetical sum of the ampl tudes of the partial" reactions produced by successive stimulations, as seen in Fig 1

In systematic series of measurements it was found that the amplitude of the combined" reaction was on a erage slightly smaller than the sum of the amplitudes of the corresponding "partial" reactions. This occlusion (calculated as the ratio of the difference between the sum of the amplitudes of the "partial" reactions and the amplitud of the corresponding combined" reaction to the sum of th amplitudes of the partial" reactions) was statistically significant (differed significantly from zero) only when supramaximal stimulation intensity was used (magnitude of occlusion 5.3 per cent, S.D 3.8 per cent, n = 25) It could therefore probably ha e been due to spread of current to the umplit nerve trunk (*cf* methods) It may hence be concluded that the summation of the partial" reactions pproximately follows the algebraic princip1 regardless of the amplitudes of the reactions.

6. *The Depend e of the Amplitud of the GSR on th Prio Neu o-S domotor Actu ty* If th stimulation of the sciatic nerve was preceded by prolonged inactivity of the peripheral neuro-effector system — as was the case owing to a lack of induced and spontaneous activity d ring the time of preparation after cutting the sciatic nerve — the amplitudes of the first GSR s were low usually of the order of 1 mV regardless of the timulation intensity During a succession of relatively frequent single stimulations, the amplitude of the GSR increased nd finally rose to a certain maximal value the same occurred after tetanisation of short duration. This post tetanic increase n the ampl tude of the GSR following a period of rest was often associated with a change in the skin d c potential (Lang 1967a) After high frequency stimulation over sufficiently long period, the amplitude of the GSR decreased again When the stimulation was now hanged to one of very low frequency the above described changes in the amplitude of the GSR took place in the opposite order

Thus, the ampl tude of the GSR evoked by a stimulation of constant intensity depends significantl on the prior induced acti ty In order *that the amplitude of* the GSR remain constant t constant stimulus intens ty it is necessary to ma

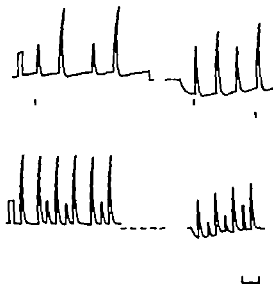


Fig. 4. Post-tetanic increase (upper trace) and post-tetanic decrease (lower trace) in the amplitude of the GSR. Both recordings were made in the same preparation using one large recording electrode applied to the central pad. Stimulation of two separate nerve slips with single impulses from two stimulators is indicated as in Fig. 1. The tetanisation periods are indicated by thick horizontal bars. The intensity and pulse duration were the same for the single impulses and tetanic trains, and the frequency of tetanisation 10 cps. Tetanic GSRs were removed from the traces. Calibrations 2 mV and 10 sec.

a sufficient constant activity in the neuro-effector system between the applied test impulses. This "basal activation" is effected also by a sufficiently great spontaneous activity (*cf.* Lang 1967a).

Fig. 4 shows the post-tetanic increase (upper trace) and post-tetanic decrease (lower trace) of the GSR, both in the same preparation. Successive stimulation of two separate nerve slips produces two GSRs of different amplitude. A relatively short tetanisation applied to one slip after a weak prior nerve activity leads to an increase only in the amplitude of the GSR corresponding to the slip that was tetanised, while the GSR evoked by stimulation of the other slip remains unchanged. Prolonged tetanisation of either slip leads to a decrease in the amplitudes of the GSRs evoked by stimulation of both slips.

Moreover, the post-tetanic increases and decreases in the amplitudes of the GSRs differed also quantitatively: when the amplitudes increased, the ratio of the maximal and minimal amplitudes of the GSR was of the order of tens, whereas the ratio was at most 2—3 when the amplitudes decreased.

Discussion

The potentials induced by stimulation of the sympathetic trunk and recorded in the distal parts of the sweat channels of a cat with micropipette electrodes are equal in polarity and amplitude and also identical in shape, when the stimulation is of short duration, to the rapid negative phase of the endosomatic GSR recorded with macroelectrodes applied to the skin (Shaver, Bruslow and Cooke 1962). The latter reaction is evidently a composite of the numerous potential events of a non-propagated graded type which are produced by cells in the wall of the distal epidermal part of the sweat channel. This effector cell group of one sweat channel

we shall refer to as a unit potential generator (UPG) and the potential they produce, which can be recorded from the sweat channel with a micropipette electrode the unit potential (UP) of the sweat gland.

If the fields of the UP's were to spread diffusely through the surrounding tissue the amplitude of the GSR recorded with a macroelectrode in different areas of the volume conductor would depend on the strength of the composite potential field Two observations deny the existence of such a volume conduction. The UP cannot be recorded when the tip of a micropipette electrode is in contact only with intact epidermis some distance away from the orifice of the sweat channel (Shaver Brusilow and Cooke 1962) Secondly an artificial local defect in the area of the epidermis under a macroelectrode causes the amplitude of the GSR to fall to a fraction of its original value The defect increases the conductance between the electrode and tissue, and this should in the case of volume conduction tend to increase the recorded signal.

The sweat gland has been considered to represent in principle a core conductor which consists of resistive components in series and parallel, and a longitudinal shunting arm, the relatively low-ohmic sweat column (Lloyd 1960) According to Lloyd the transversal resistance of the epidermis is determined by the lengths of the sweat columns in the core conductors. The longitudinal (horizontal) resistance of the epidermis itself is, however very high (Lloyd 1960 Edelberg 1966) It is obvious then that when the sweat channel is filled with sweat, the greater part of the currents generated by UPG's passes through the sweat channels which latter may be considered as extensions of the surface electrode to the UPG's. We are inclined to assume that *the negative phase of the endosomatic GSR is a composite potential which rise in complex circuit of resistive elements and UPG's the latter being coupled parallel to each other and in series with the surface electrode*

We cannot, however disregard the part played by the epidermis in this circuit. Actually its parallel resistance loading the UPG's is relatively large compared with the total serial resistance of the sweat channels if the sweat channels are filled with sweat, if the number of sweat channels under the recording electrode is large and if the epidermis is intact. A significant question is naturally whether the conductance of the epidermis remains constant when the UPG's are activated.

It is possible to explain some of the experimental observations on the basis of the presented hypothetical circuit If a part of the UPG's in the skin area where a macroelectrode is located remain inactive when the GSR is recorded, the sweat channels of these inactive glands load the unit potential generators in series with them. If the ratio of active to inactive UPG's remains constant in different skin areas the load of the active UPG's will not increase although the area of contact under the recording electrode is increased. In such cases the amplitude of the recorded GSR will not change (Fig. 2, upper traces) When, however the electrode is extended over skin areas containing only inactive UPG's, the amplitude of the GSR will decrease (Fig. 2, lower traces) Similarly, the loading of the UPG's increases and the amplitude of the GSR decreases if the electrode is in contact with

large skin areas that do not contain sweat glands (as when the whole pad is immersed in the electrolyte) or if a minor lesion greatly increases the conductance between the electrode and the subepidermal tissue. Advantage may be taken of this effect to remove artefacts caused by the GSR when other biopotentials are being recorded (Shachel 1959)

The post-tetanic increase and decrease of the amplitude of the GSR (Fig 4) are more difficult to explain. If we still consider that the UPGs are connected in parallel to each other and in series with the surface electrode and that the resistive elements of the circuit largely determine the amplitude of the recorded composite potential, we may propose the following explanation. Sweat is known to be reabsorbed from the sweat channels during periods of inactivity and as a sign of this the transversal resistance of the skin increases (Lloyd 1959 1960) This means a pronounced increase in the resistance component in series with the UPG's, and as the parallel loading resistance of the epidermis remains constant, this leads to a relative increase in the loading of the UPG's and a decrease in the amplitude of the GSR. When the sweat glands become filled with sweat after a short tetanization, the conditions are re-established. The post tetanic increase in the amplitude of the GSR will, however be observed only in the reaction associated with the tetanized nerve slip only those sweat glands innervated by fibers from this slip may become filled with sweat (Fig 4 upper trace)

Tetanisation of longer duration causes the sweat gland channels to be filled and then the serial resistances of the UPG s are minimal. The amplitude of the GSR may nevertheless decrease if the conductance of the epidermis increases Edelberg (1961 -66) found that the conductance of the epidermis may increase considerably when GSR is recorded. If such a change in conductance occurs also in the epidermis the foot pad of a cat as a result of tetanisation of the efferent nerve fibers, it ould explain not only the post tetanic decrease in the amplitude of the GSR. but also hy this decrease is not limited solely to the reaction associated with the tetanised nerve sl p The ncrease in conductance could be expected to load equally all UPG in contact with the electrode (Fig 4 lower trace)

The changes in the amplitude of the GSR caused by tetanisation can be explained without assuming ny essential influence of changes in the resistance components. The post-tetanic increase of the GSR may be the result of for instance post-tetanic potentiation of the neuro-effector transmission mechanism. A long tetanisation period may effect a strong soconstriction which weakens the activity of the effector cells in large skin areas In estig tions with micropipette electrodes are certainly required to determine at least which h pothesis is incorrect.

If our circuit hypothesis orrect the increase in the amplitude of the GSR produced by increasing the i i ion intensity may be due either to an increased density of active elements under the electrode or to an increased output of the UPG's. According to Wang 19 th weat glands are activated in an all-or nothing manner in support of which Wang presented the observation that the amplitude of the GSR undergoes a tepwise increase when the intensity of the timu-

lation is slowly increased. According to this view the amplitude increase is solely due to activation of new units. We have not, however, been able to confirm Wang's observation. Moreover, it has been shown that the amplitude of the UP recorded with a micropipette electrode in the sweat channel increases with increasing intensity of stimulation (Shaver, Brusilow and Cooke 1962). Evidently nerve fibers from several postganglionic neurons must converge on each UPG. An analogous situation prevails in the submaxillary gland where each gland cell is supplied by at least 5—10 neurons according to empirical observations (Lundberg 1955). It is not known how many postganglionic nerves supply a sweat gland, but qualitatively its nerve supply does not differ from that of other autonomic effectors (John 1940). However, the observation that in some cases where only a limited group of postsynaptic fibers is stimulated the activity evoked may be restricted only to a part of the effector area supports the view that an increase in GSR amplitude may also correlate with recruitment of new sweat glands.

The observation that the spatial summation of the amplitude of the GSR takes place mainly by the algebraic principle may be of practical value in the study of the central regulation of the GSR. The value of this information is, however, restricted by the observation that the increase in the amplitude does not follow this principle when the stimulation is repetitive, i.e. when temporal summation takes place. It would be of interest to know the nature of the neural activity combined with the galvanic skin reflex.

The fusion of the reactions caused by tetanic stimulation has been interpreted as a consequence of the inertia of the potential-producing mechanism of the sweat glands. The point of comparison has been the mechanical reaction of muscles to stimulation of motor nerves (Patton 1948). However, the fact that the increase of the amplitude of the GSR with increasing tetanisation frequency is greater the weaker the stimulation is speaks in favour of summation already at the pre-effector level. The intensity and frequency of the stimulation thus seem to be (at least qualitatively) interchangeable. This principle, known as the Cannon-Rosenblueth theory, has been found to apply to most autonomic effectors also quantitatively. Hillarp (1960) has ascribed this to the functional organization of the autonomic neuro-effector junctions: not only do branches from several postganglionic nerves converge on the same effector cell or cell group, but there also occurs overlap in the innervation structure.

All the factors in the effector mechanism that influence the amplitude of the endosomatic GSR have not been discussed here. Such factors as temperature, blood circulation and the nature and concentration of the contact electrolyte (Edelberg, Greiner and Burch 1960) may be significant.

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The Effect of Protriptyline on the Metabolism of Dopamine and Noradrenaline in Rabbit Brain in vitro

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Abstract

JONASON, J. and C. O. RUTLEDGE. *The effect of protriptyline on the metabolism of dopamine and noradrenaline in rabbit brain in vitro.* Acta physiol. scand. 1968. 73. 161—175.

Slices of rabbit brain cortex were incubated with labeled dopamine or noradrenaline. Dopamine was deaminated twice as fast as noradrenaline. Pretreatment of rabbits with protriptyline reduced the catabolism of dopamine and noradrenaline to values of 58 and 35 per cent of normal, respectively, using the same substrate concentration. Furthermore protriptyline-pretreatment inhibited the retention of each amine correspondingly, whereas the monoamine oxidase activity of brain cortex homogenates was unchanged. These observations suggest that protriptyline inhibits the metabolism of catecholamines by inhibiting the amine uptake by the cell membrane. Protriptyline (10 mg/kg) caused a marked reduction of dihydroxyphenylacetic acid, a slight reduction of homovanillic acid and an increase in methoxytyramine using dopamine as substrate, whereas a marked reduction of dihydroxyphenylglycol and dihydroxymandelic acid, a smaller reduction of both vanilmandelic acid and 3-methoxy-4-hydroxyphenylglycol with no effect on normetanephrine was seen using noradrenaline as substrate. These observations indicate that monoamine oxidase is localized at least partially intraneuronally whereas catechol-O-methyltransferase is localized almost exclusively extraneuronally. Protriptyline-pretreatment inhibited the total synthesis of noradrenaline from dopamine, an effect antagonized by increasing the substrate concentration supporting the view that dopamine-β-hydroxylase is localized within the monoamine neurons in the cerebral cortex.

The enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) are known to play important roles in the catabolism of dopamine (DA) and noradrenaline (NA) in the central nervous system (Axelrod, Albers and Clementi 1959, Rosengren 1960, Crout, Creveling and Udenfriend 1961, Carlsson and Hillarp 1962, Mannarino, Kirshner and Nashold 1963, Glowinski, Kopin and Axelrod 1965). It is also well established that dopamine β-hydroxylase (the enzyme converting DA to NA) is important in the metabolism of DA in the brain (Udenfriend and Creveling 1959, Carlsson 1959, Glowinski, Axelrod and Iversen 1966, Merritt and Schultz 1966).

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Although the localization of these enzymes in peripheral tissues has been investigated, there is little direct evidence as to their localization in the central nervous system. In sympathetically innervated organs, dopamine β-hydroxylase is considered to be localized in storage particles which are located within the adrenergic neuron (Potter and Axelrod 1963 Iversen, Glowinski and Axelrod 1966) COMT is thought to be localized outside the neuron in both peripheral tissues (Kopin and Gordon 1962) and brain (Carlsson and Hillarp 1962) MAO on the other hand, is localized both within the neuron and outside the neuron in sympathetically innervated tissues (Snyder Fischer and Axelrod 1965 Almgren *et al.* 1966)

The activities of these three enzymes have recently been investigated in our laboratory by incubating brain cortical slices with labeled DA and NA and quantitating the amounts of the products formed (Rutledge and Jonason 1967) It was shown that DA was converted to the following compounds (in the order of quantitative importance) 3 4-dihydroxyphenylacetic acid (DOPAC) homovanillic acid (HVA) methoxytyramine (MTA) and NA. The two alcohols, 3 4-dihydroxyphenylethanol (DOPET) and 3-methoxy-4-hydroxyphenylethanol (MOPET) were formed only in negligible quantities. When the slices were incubated with labeled NA, the products were (in the order of quantitative importance) 3,4-dihydroxyphenylglycol (DOPEG) 3-methoxy-4-hydroxyphenylglycol (MOPEG) 3 4-dihydroxymandelic acid (DOMA) normetanephrine (NM) and vanillmandelic acid (VMA)

Another important metabolic function of brain tissue is its ability to concentrate exogenously administered catecholamines (Dengler Spiegel and Titus 1961a, b (Ross and Renyi 1964 Hamberger and Masuoka 1965) An important concentrating echanism is localized at the level of the neuronal cell membrane and has been n histochemically to occur in brain neurons (Hamberger and Masuoka 1965) ell as in neurons in sympathetically innervated organs (Hillarp and Malmfors 964) It has been demonstrated by both histochemical and biochemical methods that this concentrating mechanism can be inhibited in the central nervous system neurons by a group of drugs which include imipramine, desmethylimipramine, and protriptyline (PTP) (Dengler Spiegel and Titus 1961b, Carlsson *et al.* 1966, Häggendal and Hamberger 1967) PTP has been found to be among the most potent inhibitors of this mechanism in peripheral tissues (Carlsson and Waldeck 1965) and brain (Carlsson *et al.* 1966)

The present study was conducted to investigate the action of PTP pon the metabolism of DA and NA in brain slices in an attempt to determine the relative importance of intraneuronal versus extraneuronal acti ity of the three enzymes involved in the metabolism of the exogenously added amines.

Methods

Adult rabbits weighing between 1 8 and 2 5 kg were used in this study Some of the animals were pretreated with either 1 or 10 mg/kg of PTP by i. injection 45 min prior to death. Eight slices of rabbit cerebral cortex approximately 225 mg) were preincubated in 3 ml of Krebs-Henseleit solution for 10 min at 37 in 95 per cent O_2—5 per cent CO_2 atmosphere (Rutledge and Weiner 1967) Then either 576, 5760 or 57 600 × 10 moles of C[1] -DA

(2.75, 7.5 or 275×10^{-9} curies respectively) were added and the flasks were incubated additional 30 min. In another series of experiments 5760×10^{-12} moles of H^3 NA (5.27×10 curies) were added instead of C DA. Control samples were prepared by the addition of 2 ml of 2 N HCl to the flask before the preincubation period. All incubations were terminated by the addition of 2 ml of 2 N HCl.

Determination of etention f the amines

The samples were incubated as described abov and after the 30 min incubation, the slices were removed and dried upon piece of filter paper. They were then reincubated for 10 min in substrate-free Krebs-Henseleit solution. In preliminary experiments t was observed that the decrease in radioactivity had reached plateau after 10 min of postincubation. The slices were then removed, dried upon piece of filter paper and added to 2 ml of 2 N HCl. The samples were homogenized, centrifuged and the sediment was re-extracted with an additional 2 ml of 2 N HCl. After the second homogenization and centrifugation, the combined super natants were filtered. The volumes of the filtrates were reduced to dryness in a freeze-drier. Liquid scintillation fluid was added and the total radioactivity determined in Packard Tri-carb liquid scintillation counter.

Isolation of NA DA d th combined acid and neutral atabolites

The separation procedures used in this study have been previously described in detail (Rutledge and Jonason 1967) and thus only general outline of these procedures will be presented here. Immediately after the termination of the incubation, 20 μg each of NA and DA were added to the flasks as carrier substances and for the determination of the recoveries throughout the separation procedure. The amines, NA and DA, were adsorbed onto and then eluted from Dowex 50, whereas the combined acid and neutral catabolites passed through in the effluent and were then extracted into ether. The recovery of the amines was determined by measuring the fluorescence of the Dowex luates and comparing this with the fluorescence of the total amount of amine added as carrier substance. The olumes of both the amine and catabolite fractions were reduced under acuum scintillation counting fluid was added and the radioactivity determined in liquid scintillation counter.

Separatio and quantitation of each of the acid and neutral atabolites

Upon termination of the incubation, 200—500 μg of each substance were added as carrier substances and for the determination of the recoveries. The samples were then extracted and the HCl extract was passed over Dowex 50 as described above. The effluents from the Dowex 50 columns were then passed over alumina to separate the catechol derivatives (which were absorbed) from the O-methylated derivatives (which passed through in the effluent). The eluates of the adsorbed material and the effluents were acidified the catabolites were then extracted into ethyl acetat. The olumes of these fractions were reduced under vacuum and the catabolites further separated by either paper or thin-layer chromatography. Each substance was then eluted from the chromatogram and portion of the eluate was used for determination of the recovery by the phenol reaction (Barness *et al.* 1963). Most of the eluate, however was reduced to dryness, liquid scintillation fluid was added and the radioactivity was determined by liquid scintillation techniques.

S paration and quantitatio of NM and MTA

These amines were determined in experiments separate from those described above. Upon termination of the incubation, 20 or 30 μg of each amine was added to each sample and the samples were extracted with 2 N HCl. The O-methylated amines were separated from their catecholamine precursors by passing the extracts through an alumina column to which NA and DA were adsorbed. The effluents from alumina, containing the O-methylated amines, were then passed over Dowex 50 and NM and MTA were then eluted in separate fractions. The recovery and radioactivity were determined as described for DA and NA.

Determ atio of MAO activity in brai ort homogenat

MAO activity was determined by method from Almgren *et al.* 1966. C^{14}-tyramine was incubated with homogenates of rabbit brain cortex and the C^{14}-phenolic acids formed were extracted into toluene. Scintillation fluid was added to portion of the organic phase and the radioactivity was determined by liquid scintillation counting techniques.

The values of DA, NA and the individual catabolites from the tissue plus the media w re calculated in terms of moles $\times 10^{-12}$ per flask obtained after a 30 min incubation period. Th alues were calculated by correcting for efficiency recovery aliquot factors, and the activity of the precursor. The alues of the combined acid and neutral catabolites as

TABLE I The effect of protriptyline (PTP) upon th metabolism of C DA in rabbit brain cortex Rabbit brain cortex slices wer incubated for 30 min with either 576, 5760, or 57 600 × prior to killing the animal. Control corresponds t samples in which 2 ml of 2N HCl Recovery of noradrenalin (NA) equals 61 7±1.5 % and that of dopamine (DA) catabolites were corrected for recovery n represents the number of experiments. S.E.M. amine or catabolite in the media plus that in the tissue Values in parentheses represent equivalent to 5 counts per minute bov background in the original sample, assuming

Treatment		576×10^{-12} moles DA		
		C NA	Acid and Neutral Catabolites	C^{14}-DA
Normal	Mean	86	133	336
		(70)	(127)	
	S.E.M.	13	8	12
	n	4	6	3
PTP 1 mg/kg	Mean	42	119	381
		(26)	(113)	
	S.E.M.	5	27	47
	n	4	5	5
PTP 10 mg/kg	Mean	17	90*	464
		(1)	(84)	
	S.E.M.	4	8	28
		6	5	[illegible]
[illegible]	Mean	16	6.0	631
	S.E.M	7	0 1	130
		3	2	2

the alues for the retention of the amines were calculated in the same manner with the exception that no recovery alues could be assessed thus no correction for this ariable was made It was assumed that the recoveries of these two groups of compounds was relatively constant Results are generally expressed as the mean ± standard error of the mean (S.E.M.) Statistic l calculations were performed according to Davies (1949) and Snedecor (1956)

Results

The ff ct of PTP pon th m tabolism of DA and NA

Incubation of normal rabbit brain cortex slices with three different substrate concentrations of DA result in the formation of quantities of acid and neutral catabolites which ar directly related to the amount of substrate employed (see Table I) This indicates that MAO is not saturated over this 100-fold range of substrate concentration. Pretreatment of rabbits with 1 mg kg of PTP had an effect upon the catabolism of DA nly at the middle substrat concentration. However when the dose was ncreased to 10 mg kg there was a reduction in the total catabolism of DA at all 3 onc nt tions of the substrate (Table I) The values of the acid and neutral catabolites obtained after PTP 10 mg/kg are from 58 to 75 per cent of the normal values depending pon the concentration of the substrate con-

slices.
10^{-12} moles of C^{14}-dopamine (2.75 27.5, or 275 × 10^{-8} curies) Protriptyline was injected 45 minutes
were added to the incubation fluid before the incubation.
equals 69.6 ± 2.4 per cent. The NA and DA values but not the values of the acid and neutral
represents the standard error of the mean. Values are presented as moles × 10^{-12} and represent
values in which the control alues ha been subtracted. 1 × 10^{-8} moles are approximately
100 per cent recovery Significantly different from normal values t $p < .05$ $p < 01$ $p < .001$

5760 × 10^{-12} moles DA			57 600 × 10^{-12} moles DA		
C^{14} NA	Acid and Neutral Catabolites	C^{14} DA	C^{14} NA	Acid and Neutral Catabolites	C^{14}-DA
154	1470	5030	301	11500	39800
(102)	(1448)		(168)		
5	66	91	56	559	998
16	7	29	6	7	7
174	1060*	3800	302	11100	40700
(122)	(1058)		(170)		
17	58	213	43	1020	2250
11	9	11	7	7	7
92	863*	4350**	301	8600*	39500
(40)	(841)		(169)		
8	57	184	69	888	2030
12	13	13	7	7	7
52	22	6050	152	28	49600
11	10	250	16	7	3080
5	3	9	2	2	3

ployed. This reduction in total catabolites is correlated with an increase in the uncatabolized DA in the two lower DA concentrations. An increase at the largest DA concentration would be difficult to detect since the expected increase is not much larger than the S.E.M. A Lineweaver Burk plot of the data demonstrated a competitive type of inhibition.

When NA was employed as the substrate much smaller amounts of acid and neutral catabolites were detected (compare normal values at the middle DA substrate concentration in Table I with those of NA in Table II) This is partly a reflection of the fact that the major NA catabolites are phenolic glycols (Rutledge and Jonason 1967) and are not extracted into ether as well as the major catabolites of DA which are phenolic acids. However a comparison of the total catabolism of NA and DA can be seen in experiments in the latter part of the study n which th odividual catabolites were isolated and corrected for recovery (T ble IV—VI) From these data it can be calculated that the total deaminated catabolites amount to 117 10 $^{-12}$ moles for DA and 903 × 10^{-12} moles for NA. When the newly formed amines are included this yields a total metabolism of 2357 10 $^{-12}$ moles for D

TABLE II The effect of protriptyline (PTP) upon the metabolism of H³ NA in rabbit brain cortex slices

Rabbit brain cortex slices were incubated for 30 min with 5760×10^{-12} moles of H³ noradrenalin ($5.27 \times 10^{-}$ curies) Recovery of noradrenaline (NA) equals 65.4 ± 2.1 per cent. The NA values but not the values of acid and neutral catabolites were corrected for recovery significantly different from normal values t $p < 0.05$

	Treatment	Acid and Neutral Catabolites	H³ NA
Normal	Mean	317	4530
	S.E.M.	55	43
	n	4	4
PTP 10 mg/kg	Mean	117	5420
	S.E.M.	17	508
		4	4
Control	Mean	10.0	5730
	S E.M	1 1	564
		2	2

1113×10^{-12} moles for NA. Thus the deamination as well as the total metabolism of NA is only about one half that of DA. This agrees with the comparison of the unmetabolized DA (middle substrate concentration) in Table I (2000×10^{-12} moles less than control) to the uncatabolized NA in Table II (1200×10^{-12} moles less than control) PTP 10 mg/kg had a marked effect on the catabolism of NA (Table II) The amounts of acid and neutral catabolites formed after pretreatment with PTP were only 35 per cent of the normal values while the unmetabolized NA s correspondingly increased. Thus the effect of PTP on the metabolism of NA (5 per cent of normal values) was much greater than the effect on DA (58 per cent of normal values) when the same concentration of substrate was used (5760×10 moles) When the per cent decrease from normal of these two groups were analyzed by an elementary analysis of variance (Davies 1949) it was found that the difference was significantly different at $p < 0.05$

Th ff ct of PTP upon th n t synthesis of NA

Net synthesis of NA occurs at all three DA substrate concentrations (Table I and Fig 2) The rat nthesis is pparently at the plateau portion of the substrate-velocity curve for the NA synthesizing system since a 100-fold increase in the substrate concentration result in only a doubling of the NA formation.

Pretreatment of the rabbits with PTP 10 mg kg results in total inhibition of net synthesis at the lowest DA substrate concentration, a 60 per cent reduction at the middle DA substrate concentration and no inhibition at the highest DA substrate concentration (Table I and Fig 2) PTP 1 mg kg results in a 63 per cent inhibition at the lowest DA substrate concentration with no inhibition at the other two DA

Net synthesis represents only uncatabolized newly formed noradrenaline

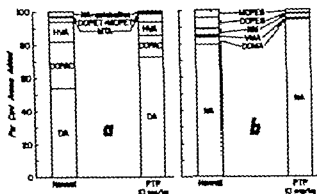


Fig. 1 Metabolism of dopamine (DA) and noradrenaline (NA) in rabbit brain cortex slices, normally and after protriptyline (PTP) pretreatment. PTP 10 mg/kg was injected 45 min prior to killing the animal. All experimental values were corrected by subtracting the control values.

a. Distribution pattern of DA and its metabolites expressed as per cent of total DA added.

b. Distribution pattern of NA and its metabolites expressed as per cent of total NA added.

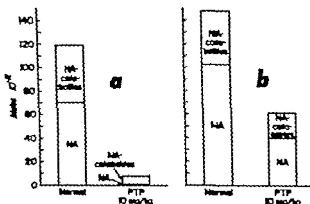


Fig. 2. The effect of protriptyline (PTP) upon the synthesis of noradrenaline (NA) from dopamine (DA) in rabbit brain cortex slices. PTP 10 mg/kg was injected 45 min prior to killing the animal. All experimental values were corrected by subtracting the control values. The values are expressed as moles $\times 10^{-12}$ of NA formed.

a. Brain slices were incubated with 576×10^{-12} moles of DA.

b. Brain slices were incubated with 5760×10^{-12} moles of DA.

substrate concentrations. These calculations were based upon the values in which the control values were subtracted. The competitive nature of this inhibition was also seen when the results were plotted according to the method of Lineweaver and Burk.

The effect of PTP upon the retention of DA and NA

From Table III it can be seen that DA and NA are retained to approximately the same extent when the slices are incubated with 5760×10^{-12} moles of amine. When the rabbits were pretreated with PTP 10 mg/kg the values of DA retained were reduced to 66 per cent (576×10^{-12} moles DA) and 81 per cent (5760×10^{-12} moles DA) of the normal values. These values were calculated after the control values had been subtracted. PTP had no effect when the slices were incubated with $37\,600 \times 10^{-12}$ moles of DA. PTP 1 mg/kg had no measurable effect upon the retention of DA at any of the 3 substrate concentrations.

When the slices were incubated with 5760×10^{-12} moles of H^3-NA, 10 mg/kg of PTP reduced the values of NA retained to 29 per cent of the normal values (with control values subtracted). Thus PTP had a much greater effect upon the retention

TABLE II The effect of protriptyline (PTP) upon the metabolism of H³ NA in rabbit brain cortex slices

Rabbit brain cortex slices were incubated for 30 min with 5760×10 moles of H³ noradrenaline (5.27×10^{-4} curies) Recovery of noradrenaline (NA) equals 65 4±2.1 per cent. The NA values but not th values of acid and neutral catabolites were corrected for recovery significantly different from normal values at $p<0.05$

	Treatment	Acid and Neutral Catabolites	H³ NA
Normal	Mean	317	4530
	S.E.M.	35	43
	n	4	4
PTP 10 mg/kg	Mea	117	5420
	S.E.M.	17	308
		4	4
Control	Mean	10.0	5730
	S E.M.	1 1	564
		2	2

1113×10^{12} moles for NA. Thus the deamination as well as the total metabolism of NA is only about one-half that of DA. This agrees with the comparison of the unmetabolized DA (middle substrate concentration) in Table I (2000×10^{12} moles less than control) to the uncatabolized NA in Table II (1200×10^{12} moles less than control) PTP 10 mg/kg had a marked effect on the catabolism of NA (Table II) The amounts of acid and neutral catabolites formed after pretreatment with PTP were only 35 per cent of the normal values while the unmetabolized NA correspondingly increased. Thus the effect of PTP on the metabolism of NA 5 per cent of normal alues) was much greater than the effect on DA (58 per cent of normal values) when the same concentration of substrate was used ($5760\times$ 10 moles) When the per cent decrease from normal of these two groups were analyzed by an elementary analysis of ariance (Davies 1949) it was found that the difference was significantly different at $p<0.05$

Th effect f PTP upon th n t ynthesis of NA

Net synthesis of NA occurs at all three DA substrate concentrations (Table I and Fig 2) The rate ynthesis is apparently at the platea portion of the substrate-velocity curve for the NA synthesizing system since a 100-fold increase in the substrate concentration results in only a doubling of the NA formation.

Pretreatment of the rabbit w th PTP 10 mg kg results in total inhibition of net synthesis at the lowest DA ubstrat concentration, a 60 per cent reduction at the middle DA substrate concentr tion and no inhibition at the highest DA substrate concentration (Table I and F g PTP 1 mg kg results in a 63 per cent inhibition at the lowest DA substrate con entration with no inhibition at the other two DA

1 Net synthesis represents only unc tabolized newly formed noradrenaline

TABLE IV The effect of protriptyline (PTP) upon the detailed catabolism of C[14] DA in rabbit brain cortex slices

Brain cortex slices from normal and PTP pretreated rabbits were incubated with either 576 or 5760 × 10⁻¹¹ moles of C^{14} DA (2 75 or 27.5 × 10⁻⁴ curies) Mean recoveries of the catabolites based upon 11—55 values dopamine (DA) 69.6 ± 2.4 (per cent) 3,4-dihydroxyphenylacetic acid (DOPAC) 25.5 ± 1.2, homovanillic acid (HVA) 30.8 ± 1 9 methoxytyramine (MTA) 52 0 ± 2.2, 3,4-dihydroxyphenylethanol (DOPET) 19 1 ± 1 4 3-methoxy-4-hydroxyphenylethanol (MOPET) 15 1 ± 1 6. All values were corrected for recovery For further explanation see Tabl I significantly different from normal values at $p < 0.01$ $p < 0.001$

Treatment		DA	DOPAC	HVA	MTA	DOPET	MOPET
5760 × 10⁻¹¹ moles DA							
Normal	Mean	9050	1440	620	206	21	12
					(138)		(10)
	S.E.M.	91	70	57	10	4	9
	n	29	9	18	10	5	4
PTP	Mean	5800	852	438	170	35	12
1 mg/kg					(102)		(10)
	S.E.M.	213	27	77	26	32	2
		11	2	7	4	2	2
PTP	Mean	4350	753 **	434	348 **	29	5
10 mg/kg					(200)		(3)
	S.E.M.	184	38	59	23	18	3
		13	5	11	4	4	4
Control	Mean	6050	7	12	68	0 2	2
	S E.M	250	2	5	15	0 2	2
		9	6	8	4	3	2
576 × 10⁻¹¹ moles DA							
Normal	Mean	356	92	40	41	18	7
				(37)	(16)	(14)	
	S E M	12	1	8	12	4	4
		3	3	4	4	4	4
PTP	Mea	464	66	27	56	4	0
10 mg/kg				(24)	(31)	(0)	
	S E.M.	28	13	3	5	2	—
		6	3	3	4	4	1
Control	Mea	631	0	3	25	4	0
	S E M.	150	0	1	11	4	0
		2	2	2	2	2	2

Th effect of PTP upon th total synthesis of NA

The total synthesis of NA was calculated as the total of C NA plus the 5 C catabolites of NA (NM DOMA, VMA, DOPEG and MOPEG) formed from C[14] DA. From Table V and Fig. 2 it can be seen that PTP markedly inhibits the total synthesis of NA. When the slices were incubated with 576 × 10⁻¹¹ *moles of*

TABLE V The effect of protriptyline (PTP) upon the detailed catabolism of newly-formed C^{14}-NA in rabbit brain cortex slices

Brain cortex slices from normal and PTP pretreated rabbits were incubated with either 576 or 5760×10^{-11} moles of C^{14} DA (2 75 or $27.5 \times 10^{-}$ curies) Mean recoveries of the catabolites based upon 18 to 104 values: noradrenaline (NA) (in per cent) 61 7±1.5, 3 4-dihydroxymandelic acid (DOMA) 13 4±1 4 3-methoxy-4-hydroxymandelic acid (VMA) 18.0±2.3, normetanephrine (NM) 70.2±4 1 3 4-dihydroxyphenylglycol (DOPEG) 10.8±0.8, 3-methoxy-4-hydroxyphenylglycol (MOPEG) 20.8±2.1 All values were corrected for recovery

Treatment		NA	DOMA	VMA	NM	DOPEG	MOPEG
5760×10^{-11} moles DA							
Normal	Mean	154	9	12	43	32	12
		(102)	(4)	(0)	(0)	(31)	(10)
	S.E.M.	5	5	3	6	7	3
	n	26	8	9	7	7	12
PTP	Mean	174	0.6	5	13	52	2
1 mg/kg		(122)	(0)	(0)	(0)	(51)	(0)
	S.E.M.	17	0 6	3	2	30	2
	n	11	4	2	2	2	2
PTP	Mean	92	3	16	40	6	18
10 mg/kg		(40)	(0)	(0)	(0)	(5)	(16)
	S.E.M.	8	4	7	24	3	5
		12	6	5	2	7	6
Control	Mean	52	5	17	62	1	2
	S.E.M.	11	2	6	1	1	1
	n	5	6	5	2	6	5
576 10^{-11} mole DA							
Normal	Mean	86	5	7	22	25	12
		(70)	(5)	(7)	(0)	(25)	(12)
	S.E.M.	13	3	2	4	—	12
		4	4	3	4	1	2
PTP	Mean	17	6	1	14	0	0
10 mg/kg		(1)	(6)	(1)	(0)	(0)	(0)
	S.E.M.	4	6	1	0.3	—	0
		4	4	3	4	1	2
Control	Mean	16	0	0	65	0	0
	S.E.M.	7	—	0	11	0	0
		3	1	2	2	2	2

only metabolites of NA could be detected after pretreatment with PTP 10 mg/kg The total synthesis of NA was only 7 per cent of normal. When the amount of DA was increased to 5760×10 moles, the total synthesis of NA was 41 per cent of normal. When the animals were pretreated with PTP 1 mg/kg with this amount of DA substrate the synthesis of NA was unaffected

TABLE VI The effect of protriptyline (PTP) upon the detailed catabolism of exogenously added H^3 NA in rabbit brain cortex slices

Brain cortex slices from normal and PTP pretreated rabbits were incubated with 5760×10^{-13} moles of H^3-noradrenaline (5.27×10^{-6} curies) Mean recoveries based upon 6–10 values noradrenaline (NA) (in per cent) 66.4 ± 2.1 3 4-dihydroxymandelic acid (DOMA) 22.6 ± 2.5 3-methoxy-4-hydroxymandelic acid (VMA) 13.3 ± 2.9 normetanephrine (NM) 36.9 ± 4.0 3,4-dihydroxyphenylglycol (DOPEG) 11.5 ± 1.8, and 3-methoxy-4-hydroxyphenylglycol (MOPEG) 27 1 ± 1 7 All values were corrected for recovery For further explanation see Table I Significantly different from normal † $p < 0.001$

Treatment		NA	DOMA	VMA	NM	DOPEG	MOPEG
Normal	Mean	4330	244	84.0	332	377	257
			(237)	(82.6)	(210)	(377)	(253)
	S.E.M.	43	39	19.8	23	80	51
		4	4	8	8	7	10
PTP	Mean	5420	34	9.2	296	28	113.3
			(27)	(7.8)	(176)	(28)	(109 6)
10 mg/kg	S.E.M	508	9	1.6	10	3	8.3
	n	4	4	3	4	4	4
Control	Mean	5730	7	1 4	122	0	5.7
	S.E.M.	564	1.2	1 4	37	0	1.8
		2	2	2	3	2	2

The effect of PTP upon the detailed catabolism of exogenously added H NA

Incubation of cortex slices with NA leads to the following catabolic products listed as the per cent of the total catabolites (after subtracting the control values) DOPEG (34) MOPEG (23) DOMA (21) NM (19) and VMA (3) The quantities of moles × 10^{-13} formed can be seen in Table VI From Fig 1b it can be seen that pretreatment with PTP 10 mg/kg results in large decreases in the amounts of DOMA, VMA and DOPEG The values of MOPEG were reduced to a smaller extent while the NM values were unaffected. The relatively large control values for NM are due to incomplete separation of NM from the large amounts of NA used as precursor

Discussion

There is accumulated evidence that catecholamines are taken up by specific nerve terminals in central nervous system tissue. Hamberger and Masuoka (1965) have employed the fluorescence technique of Hillarp and Falck to demonstrate uptake of neuroamines into nerve terminals in rat cortical slices. There is also biochemical as well as histochemical evidence that this uptake occurs at the level of the cell membrane and is inhibited by a group of antidepressive drugs which include cocaine imipramine desmethylimipramine, and PTP (Hamberger and Masuoka 1965 Carlsson *et al* 1966, Glowinski and Axelrod 1966, Häggendal and Hamberger 1 71) That PTP inhibits the uptake of amines at the cell membrane rather

intraneuronal storage site has been demonstrated by subcellular distribution studies in mouse heart (Lundborg and Stitzel 1967)

The present study also presents evidence that PTP inhibits the uptake of amines at the level of the cell membrane since the deamination (partly an intraneuronal process) of both DA and NA was decreased in brain slices with no effect on the MAO activity in homogenates of brain tissue. This decrease in catabolic activity was accompanied by a reduction in the amount of both amines retained by the tissue. This suggests that the intraneuronal MAO activity was inhibited indirectly without affecting the activity of extraneuronal MAO

It is interesting to compare the effects of PTP upon the metabolism of DA to those on the metabolism of NA. PTP 10 mg/kg reduced the acid and neutral catabolites of DA to 58 per cent while those of NA were reduced to 35 per cent, when the same substrate concentration of each amine was employed. This greater effect of PTP upon the catabolism of NA could mean that NA is taken up by the PTP sensitive uptake mechanism to a greater extent than DA and thus a blockade of this mechanism would result in a greater inhibition of NA metabolism. This is supported by the data which indicate that the amount of NA retained by the tissue is decreased by PTP to a greater extent than the amount of DA retained. It is not possible to state whether this is due to a greater affinity for the PTP sensitive uptake mechanism or whether there is a difference in the amount of substrate at the site of uptake due to differences in extraneuronal catabolism. Hamberger (1967) incubated rat cortex slices with several catecholamines and observed that DA and NA were taken up by the neuron to approximately the same extent. However it is possible that DA was taken up by another mechanism as well as by the PTP sensitive brane pump mechanism. An alternative explanation might be that NA and were both taken up to the same extent by the "membrane pump" but that the which did enter the neuron was metabolized to a greater extent than DA. This is not tenable since it has been shown that DA, not NA, is the better substrate for MAO (Blaschko Richter and Schlossmann 1937 Weiner 1960) A second alternative explanation could be that again NA and DA were taken up to the same extent by the membrane pump" but that DA was taken up by the storage granules to a greater extent than NA and thus protected from the action of MAO This would be reflected as an apparently greater effect on the metabolism of NA. However it is known that DA and NA apparently have the same affinities for the adrenal storage granules (Carlsson, Hillarp and Waldeck 1963) Thus if these conditions may be applied to the brain, it does not appear as if DA could be protected to a greater extent than NA. Therefore a tentative explanation for the greater reduction in the metabolites of NA as opposed to DA is that NA utilizes the membrane pump to a greater extent than DA However one cannot state that this is due to a greater affinity for the uptake mechanism since one cannot be assured that the substrate concentration of the two amines at the uptake site is the same

It has recently been demonstrated that the neurons located in the striatal region contain primarily DA with very little NA (Bertler and Rosengren 1959 Fuxe Hök

felt and Nilsson 1964) The neurons in the cerebral cortex, on the other hand, contain primarily NA (See Hamberger 1967) It has been demonstrated histochemically that PTP does not inhibit the uptake of catecholamines in the DA neurons of the caudate nucleus but does inhibit the uptake of amines in the NA neurons in the cerebral cortex (Hamberger 1967 Häggendal and Hamberger 1967) It has also been demonstrated in this laboratory that PTP has no effect upon the catabolism of either NA or DA in the caudate nucleus (Jonason and Rutledge, 1968) This is also consistant with the hypothesis that PTP inhibits the metabolism by inhibiting uptake at the cell membrane.

A complicating factor in the interpretation of these results is that there are apparently other cells in the brain which contain MAO and are capable of concentrating neuroamines (Hamberger and Masuoka 1965) These cells are primarily pericytes which occur in the endothelial cell walls of the capillaries. There is also evidence that this uptake can be inhibited by desmethylimipramine (Hamberger 1967) but there is additional information that the high concentrations required are not achieved when the drug is administered *in vivo* (Carlsson 1966)

The effects of PTP upon the detailed catabolism of DA and NA can be used to obtain some idea of the localization of COMT and MAO In general, it is seen that those products which were only deaminated *i.e* DOPAC DOMA, and DOPEG were reduced to the greatest extent those products which were both deaminated and O-methylated *i.e* HVA, VMA and MOPEG were reduced to a lesser extent and those products which were only O-methylated *i.e* MTA and NM were either not reduced or were actually increased. Since PTP is presumed to inhibit the uptake of the amines across the cell membrane those processes which occur within the neuron would be inhibited while those occurring outside of the neuron may actually be increased since more substrate is available. These findings are thus consistant with the view that MAO is at least partially localized intraneuronally and that COMT is localized almost entirely outside the neuron (Carlsson and Hillarp 196_, Kopin and Gordon 1962)

These results are consistant with studies in which the catabolites are formed *in vivo* Carlsson *et al* (1966) have observed that MTA is slightly increased in the brain after pretreatment with desipramine, l-dihydroxyphenylalanine, and nialamide (an MAO inhibitor) Glowinski, Axelrod and Iversen (1966) upon injecting H^3 NA intraventricularly into the brains of rats observed that the catechol deaminated metabolites were reduced, but that H^3 NM was either unchanged or slightly increased. Sharman (1966) measuring the endogenous levels of HVA, found no effect after pretreatment with desmethylimipramine or imipramine When desmethylimipramine was administered after the intracisternal injection of H^3 NA the drug had no effect upon deamination (Schanberg, Schildkraut and Kopin 1966) again suggesting that these agents do not inhibit the enzyme MAO directly De nervation studies in peripheral tissues have shown that about 1/3 of the MAO activity is located within the adrenergic neurons (Snyder Fischer and Axelrod 1965 Almgren *et al* 1966)

It was also observed in this study that PTP pretreatment resulted in a marked inhibition of the total synthesis of NA. Since it was observed with the MAO data that PTP is inhibiting uptake at the cell membrane in this preparation, it is likely that the inhibition of the formation of NA is due to inhibition of the uptake of the precursor thus reducing the concentration of DA substrate at the site of the NA synthesizing system. The reduction of quantities of newly formed NA has also been measured *in vivo* in brain (Carlsson *et al.* 1966, Glowinski, Axelrod and Iversen 1966). However it has been found that antidepressives are able to inhibit the enzyme dopamine β-hydroxylase at high concentrations administered *in vitro* (Goldstein and Contrera 1961). The possibility that these concentrations are reached when the drug is administered *in vivo* cannot be excluded at the present time. However the effect of these drugs upon the amine uptake mechanism occurs at drug concentrations 1/100 to 1/1000 of that required for inhibition of dopamine-β-hydroxylase (Hamberger 1961). It is not tenable however that the NA is formed normally but that its reuptake is inhibited by PTP as suggested by Glowinski *et al.* (1966) since the total NA formed (NA plus catabolites of NA in both the medium and the slices) was markedly reduced. The fact that NA synthesis could be inhibited to negligible quantities also is evidence that all the dopamine-β-hydroxylase occurs within the adrenergic neuron.

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Acta physiol. scand. 1968 73 176—185

From the Institute of Neurophysiology University of Copenhagen, Denmark

Excitability of the Frog's Muscle Spindle to Transient Stretches Following Spontaneous and Evoked Afferent Action Potentials[1]

By

Samia Al Azharia Jahn

Received 25 October 1967

Abstract

Jahn S. Al Azharia. *Excitability of the frog's muscle spindle t transient stretches following spontaneous and evoked afferent action potentials* Acta physiol. scand. 1968 73 176—185

I isolated muscle spindles of the frog the recovery of excitability tested by threshold stretches lasted for 30 msec (17—20 C) whether the conditioning afferent action potential occurred spontaneously or as evoked by stretch. Suprathreshold tretches were responded to earlier depending on the size and the rise time of the stretch. The minimum interval between spontaneous afferent action potential and the onset of a suprathreshold stretch responded to decreased with increasing rise time whereas the irresponsiv period was shortest for fast tches. When the afferent action potential was evoked by conditioning stretch the minimum rval between the response and the onset of test stretches responded to was longer than after pontaneous action potential, and increased with the size of the conditioning deformation. ere was evidence for period of supernormal excitability occurring about 8 msec (17—20 C) after spontaneous and an evoked afferent action potential.

Afferent tion potentials in isolated frog muscle spindles occur either in response to a stretch or w thout preceding mechanical stimulus. To investigate the effect of transient stretches on excitability paired stretches were applied and their effect compared with that of a tretch given at arying time intervals after a spontaneous action potential

Methods

Pr parat on The experime ts were performed on isolated muscle spindles of the m. extensor *longus dig IV* of *R na mporaria*. The experimental technique has been described (Jahn 1968 and b) The prepa io ontaining 1—2 mm of the isolated muscle spindle remainders of destroyed parallel spindle mps of extrafusal muscle fibres and the two tendons) was stretched 20—25 per cent abov equilibrium length, i.e. the length at which the preparation was just taut The temper ture of he Ringer bath was 17—20 C.

[1] Preliminary report Jahn (1967)

Stimulatio and recordi g One or tw rectangular pulses from stimulator (DISA Multistim) were fed in one or two pulse-shaping units t the stretch device (Jahn 1968 b) T reduce the ariation of the rise time to less than 1 per cent, the condenser of the two pulse-shaping units was charged by constant current generator Different rise times (2, 5 and 8 msec) are obtained by varying the current.

T investigate *th effect f an affer t actio potential not associated with deformatio* the test stretch was triggered by the spontaneous action potential fter arying time intervals (0.5—40 msec) Subsequent spontaneous action potentials which occurred earlier than 1.5 second after the afferent impulse under investigation were prevented from triggering the stretch device.

T investigat *th effect f afferent actio potential oked by deformat* pairs of stretches were applied 6—40 msec apart, the choice of the minimum interval depending on the total duration of the first stretch. The size of the conditioning and of the test stretch could be aried independently their rise time was the same.

The afferent action potentials were recorded with capillary microelectrodes inserted into the perifascicula space of the equatorial region of the isolated muscle spindles (Jahn 1968)

Results

1) St et h s appl d after a spo taneou fferent actio potential.

) *Diff rent at s of stretch* The excitability was expressed in terms of the minimum interval between a spontaneous afferent impulse nd the onset of th test stretch which regularly evoked an afferent response. These discharges recorded intra fusally from the equatorial region represent propagated spike potentials comparable to those recorded outside the pindle capsule from the afferent nerve (Jahn 1968 a)

When th stretch was threshold (T) the minimum interval was independent of the rise time when the stretch was suprathreshold (2×T) the minimum interval decreased with ncreasing rise time (Fig 1 Table I and II) Stretches of 3×T pplied 0.5—1 msec after a spontaneous afferent action potential only evoked responses when the rise time was 8 msec With a rise time of 2 msec the minimum interval between a spontaneous impulse and the onset of stretches effective in producing responses was 2 msec (7 spindles) only in one spindl were occasional responses observed with an interval of 1 msec (Table II) On the other hand, th minimum interval between the two afferent action potentials, the irresponsive period" ncreased w th increasing rise time of the suprathreshold test stretch (Fig A and Table I)

The latency of the response to test stretches was prolonged when the stretch was pplied less than 5 msec after a spontaneous afferent impulse (Fig. 2 B)

b) T st st et h f different si e Th minimum interval between a spontaneous fferent action potential and the onset of a stretch which evoked an afferent response as well as the irrespons period decreased with increasing deformation (F g 3 nd T ble II) When the stretch was threshold the irresponsive period was th same as the minimum interval between spontaneous afferent impulses (25—35 msec W th deformation of 3 times threshold the shortest interval between a pontaneous action potential and a response evoked by a suprathreshold tretch with rise t me of 2 msec was 5 msec Larger deformations were not tudied but with

Determined for single transient stretches (Jahn 1968 b)

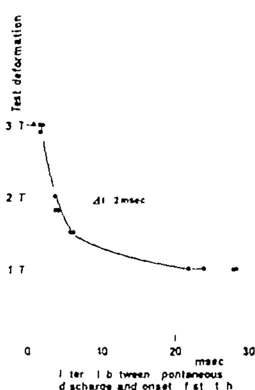


Fig 3

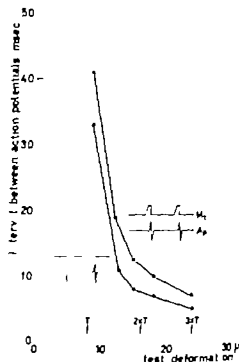


Fig. 4

Fig 3 Decrease of the terval between the spont neous action potential and the onset of tret h w th ncreasing deformation of the test tretch The fluctuations t threshold are indicated by the broken line

Fig 4 Exc tabilit afte spontaneous afferent action potential (O—O) as compared to that fter an evoked fferent response (●—) measured by he minimum interval between afferent action potentials as function of the test deformation (8 spindles, 17—20 C)
Left inset the spontaneou afferent impulse triggered the test stret h
Right inset paired st et hes of equal size
T hreshold t single tr nsien tretch
M mechan l ran ient
A ac ion potential

which stretches f a pair wit a rise time of 2 msec could be applied was 6 msec Therefore a m nim m ters l of 6 msec between responses did not represent the shortest possible interval

a) The size of th d t g t t l After a response evoked by conditioning stretches (1—3 times th eshold spontaneou afferent action potentials were not observed until 25—35 ms) l elapsed.

The minimum int rv l I twe n the responses to paired stretches increased with the size of the conditioning r t h The ncrease was most pronounced when the test stretch was 1.2—2 T d had a rise t me of 2 msec (Fig 5 B) When both stretches were threshold the m nim m interval between responses (35—43 msec)

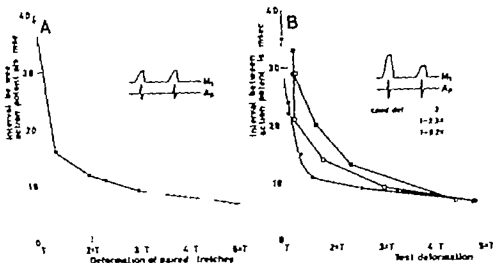


Fig 5. Impaired responsiveness after conditioning stretches of suprathreshold size measured by the minimum interval between afferent responses as function of the test deformation.
A Identical conditioning and test stretches.
B
The size of the conditioning and test stretches was aried independently (3 spindles, (18—20 C
M_t mechanical transient.
A_p action potential.

was similar to that between a spontaneous afferent action potential and a response to a test stretch (28—37 msec, Table 1 C) When the conditioning and the test stretches were two or three times threshold, the minimum interval between afferent responses was about 30 c longer than that between a spontaneous afferent action potential and the response to a test stretch of —3×T (Fig. 4)

Conditioning stretches of 4 6—5×T evoked two afferent responses. The shortest nterval between the last of them and the response to a test stretch was —9 msec longer than the shortest intervals after a conditioning stretch which evoked only a single afferent response (deformation 1—2×T Fig. 5 B)

b) Th sı.e f th test st et h A test stretch of threshold size applied more than 30 msec after a conditioning stretch evoked a response, whether the conditioning stretch was just above or five times threshold (Fig. 6 B) With 10—20 msec between stretches the test deformation required increased with the conditioning stretch (Fig 6 B and C) With 6 msec between stretches the minimum test deformation was 4 to 5 times threshold whether the conditioning stretch was threshold or above t (Fig 6 B Single stretches of 4—5 times threshold and with a rise time of 2 msec wh h evoked two afferent responses (Jahn 1968 b) only evoked a single response wh applied 6 msec after a cond tioning stretch

The latency of the response to a suprathreshold test stretch (— T pplied shortly after a conditioning stretch was 1—3 msec longer than after a singl transient tretch of the same size (repeat rate below 1/sec) as if the actual stim l s had been smaller

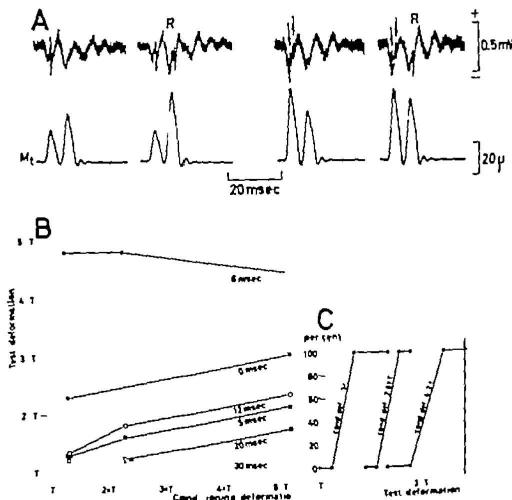


Fig 6. Responsiveness as function of the conditioning stretch (1—5×T)
A. With 6 msec between stretches the size of the critical test stretch which evoked response (R) was the same small and large conditioning deformations.
Note the two afferent spikes (↓↓) evoked by conditioning stretch exceeding 5×T (the action potentials are superimposed movement artefact)
M mechanical transient
B The critical test deformation as function of the size of the conditioning deformation with different intervals between stimuli
C. Responsiveness probability of response) to test deformation at an interval of 1 msec for different conditioning stretches
(Temp 17—20 C

3 Amplitude of afferent potentials

Usually the afferent response to the conditioning and the test stretch had the same amplitude. Only with high conditioning stretches (4—5×T) did test stretches of 3.5—4×T evoke spikes which had a 20—30 % smaller amplitude than the conditioning response. The amplitude was the same when the test deformation was increased beyond 4×T

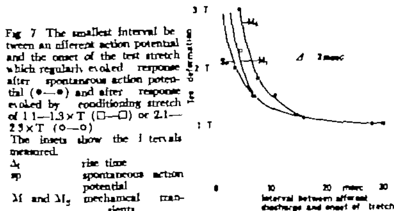


Fig 7 The smallest interval between an afferent action potential and the onset of the test stretch which regularly evoked response after spontaneous action potential (●—●) and after response evoked by conditioning stretch of 1.1—1.3 × T (□—□) or 2.1—2.5 × T (○—○)
The insets show the intervals measured.
Δ_t rise time
sp spontaneous action potential
M and M_2 mechanical transients.

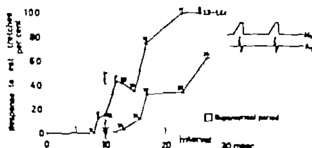


Fig. 8. Supernormal phase of excitability after an afferent response evoked by conditioning stretches of 1—1.4 × T (rise time [illegible] msec) as measured by the percentage of responses to test stretches of equal size. The interval indicates the interval between stretches as shown in the inset. The number of stimulations is indicated for the different intervals. If the change in excitability is related to the afferent action potential, supernormality occurred 7—8 msec after the evoked action potential
(5 spindles, temp 17—20° C)
M_t mechanical transient

Excitability after a spontaneous and after an evoked action potential.

When the onset of the test stretch (rise time 2 msec) which regularly evoked a response was related to the preceding afferent action potential, whether spontaneous or evoked, the minimum interval was shortest after the spontaneous impulse and increased with the size of the conditioning stretch (Fig 7) The minimum time of recovery of responsiveness to threshold stimuli (30 msec) was the same whether the decrease in excitability was caused by a spontaneous afferent impulse or conditioning stretch.

There was evidence of a supernormal period of excitability after spontaneous and an evoked action potential 8—11 msec after a spontaneous action potential and 6—8 msec after an evoked action potential the percentage of test stretches of 1—1.4 T which evoked a response when applied within this interval was greater than before and after it (Fig 8)

Discussion

The sequence of changes in excitability after an evoked afferent action potential—as studied by the recording of propagated afferent responses—was similar in isolated muscle spindles, Pacinian corpuscles and tactile receptors of the frog skin, but the different phases differed in duration. In the muscle spindle normal excitability to threshold stimuli was restored after 30 msec, in the Pacinian corpuscles after 10 msec (Loewenstein and Altamirano-Orrego 1958) in certain cutaneous receptors after 40—100 msec and in others after 2 sec (Catton 1961) In the muscle spindle a supernormal excitability occurred about 8 msec after in Pacinian corpuscles (Loewenstein 1958 b) 6—10 msec after an evoked action potential.

In the muscle spindle the shortest interval between paired stretches of ? msec rise time at which the test deformation (4—5 × T) evoked an afferent response was 6 msec. However this interval probably does not represent the absolutely refractory period because the procedure employed in this study did not allow shorter intervals between stimuli to be investigated

In Pacinian corpuscles (Loewenstein and Altamirano-Orrego 1958) the absolutely refractory period for the propagated response after a conditioning mechanical stimulus lasted 1 8—3 msec (19—26° C) tactile receptors of the frog's skin (Catton 1961) it lasted about 3 msec i e longer than the absolutely refractory period of a spike potential propagated in a myelinated axon (1.5 msec) at the same temperature (20 C) The prolonged refractory period of the receptors could be due to properties of the non myelinated portion of the sensory terminal, possibly slow impulse conduction.

In muscle pindles the latency of the afferent action potential evoked by a test was prolonged, when the test stretch was applied after an interval when only third to one half of the stimuli evoked a response

In muscle and nerve fibres this prolongation is due both to delayed firing and to slowing in conduction of the test response (Buchthal and Engbæk 1963) In recording from the equatorial region of the muscle spindle the distance of conduction is so short that the lowing hardl can contribute to the prolongation of the latency which therefor is d e ol l to delayed firing

In muscle pindles a lowered excitability during the relative refract ry period (10—20 msec after the conditioning tretch) was reflected in the 1 5—? times higher test deformatio n required to obtain a response after a conditioning tretch of 2—5 × T as compared w th that req red after a stretch of threshold size

Studies of the gener t r potential of refractory Pacinian corpuscles (Loewenstein and Altamirano-Orreg 1958 showed an increase by a factor of three in the height required to initiat propagated resp nses and a reduction in amplitude of the generator potentials during the relat refractory period. The depression of the second generator potential in reased w th the ize of the conditioning stimulus (Diamond, Gray and Inman 1958 Th increase in depression was non-linear and most pronounced for small stimuli A imila ncrease of the critical test deformation with the size of the conditioning stret h w found n muscle spindles (Fig 6)

In the Pacinian corpuscles as in the muscle spindle a spontaneous impulse did not occur subsequent to an evoked response unless a time interval had elapsed which corresponded to the recovery of excitability to threshold stimuli (10 msec Loewenstein 1958 a)

In muscle spindles the minimum interval between two afferent action potential was shorter when the first action potential occurred spontaneously than when it was evoked by a conditioning stretch.

Similarly test stimuli of the same size evoked in tactile receptors responses after 1.5—2 times longer intervals when the conditioning stimulus was mechanical than when it was electrical (Catton 1961) In Pacinian corpuscles the absolutely and relatively refractory period of the all-or-none response were the same whether the conditioning stimulus was mechanical or electrical (Loewenstein and Altamirano-Orrego 1958 and Loewenstein 1958 b)

That the depression of the response of the muscle spindle to a test stretch following a suprathreshold conditioning stretch was mainly due to the [illegible] mechanical changes is also evidenced by the prolonged depression of excitability with increasing conditioning stretch. Thus the mechanical properties of the structures connected with the sensory ending seem to affect its excitability as they determined the interval between completed stretch and the afferent response ([illegible] latency Jahn 1968 b) This assumption is consistent with the effect of [illegible] enzymes on the elasticity of the structures surrounding the sensory terminal and the excitability of tactile receptors of frog's skin (Catton and Petoe 1966

I wish to express my sincere gratitude to professor F Buchthal for [illegible] encouragement, to Poul Rosenfalck, M. Sc. for valuable theoretical discussion [illegible] Gald, E. E. and M. A. Andersen for the design of the electronic equipment.
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Acta physiol scand. 1968 73 186—195

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The Effect of Oxygen Breathing at Atmospheric Pressure on the Metabolism of Glycerol and Ethanol in Cats

By

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Abstract

LARSEN J A *The ffect of oxygen breathing at atmospheric pressure on the metaboli m f glycerol and thanol in cat* Acta physiol scand. 1968. 73 186—195

It was found ha breatl ng of oxygen may depress the limination rat of glycerol in chloralose anesthetized ts, but the depressive ffect was highly dependent on the simultaneous metabolism of ethanol. When glycerol and ethanol were infused together the addition of glycine increased he limination rate of glycerol. This increase was inhibited by oxygen eathing A simila although less pronounced change was seen I the metabolism of ethanol resul eeem that the ffect of oxygen breathing may be explained by inhibition of ydrogenases respon ble for the oxidation of ethanol and by inhibition of the production of Fosine triphosphat

After a lat nt period of varying length, exposure to pure oxygen at atmospheric pressure le id t the development of various symptoms of toxicity and, finally to the deatl of the experimental animal. The cause of the toxic effect is obscure but in-vitro experiment ha revealed that tissue respiration may be depressed by oxygen at pressures I n normal and that several enzymes are poisoned (Dickens 1946a, b Dickens 196 Da ies and Davies 1965 Lambertsen 1965) However so far it has not been pos H n intact rganisms to demonstrate any metabolic change which could account I r th f tal effect of xygen.

During tudies f the n tabolism of glycerol and ethanol it was noticed that breathing of oxygen depr sed th elimination rate of glycerol This effect of oxygen was f rther invest gated a id tl s rticl is a report on experiments on cats, in which the elimination rate of gly erol nd ethanol under certain metabolic conditions is depressed immediately and pparently reversibly after breathing of oxygen at atmospheric pressure

[1] Part of this study was performed t he Ins it t of Physiology U iversity of Copenhagen.

Methods

The cats were anesthetized with chloralose (70 mg/kg). They were kept in huge cages which allowed a high degree of physical activity and were given a standard diet consisting of boiled fish and milk. 1 hrs prior to the experiment food was withdrawn, but the cats had free access to water. A femoral artery was cannulated and used for blood sampling and blood-pressure recording. A femoral vein was used for the infusions. The temperature was kept constant at 38° C, and blood pressure, pulse rate and respiration were followed. Unless otherwise stated, the ureters were ligated through a retroperitoneal incision in order to avoid loss of infused substances through the kidneys.

Administration of oxygen

Humidified oxygen was administered either by flushing the horizontal limb of a T-cannula inserted into the trachea with oxygen at a rate of 2—3 l/min or by flushing vigorously with oxygen a beaker fitted over the head of the cat.

Administration of glycerol and ethanol and calculation of elimination rate

The enzymes involved in the initial steps of the break-down of glycerol and ethanol are mainly localized in the liver (Larsen 1963 a, b) and are saturated at arterial concentrations of approximately 3 mM for glycerol and 1 mM for ethanol. The enzymes situated in extrahepatic organs are saturated at even lower concentrations. This means that above these concentrations of glycerol and ethanol the elimination rate is constant and independent of the concentration and also within wide limits independent of blood flow. A priming dose was given in order to rise the glycerol concentration to about 7 mM and the ethanol concentration to about 3 mM. The priming dose was dissolved in 20 ml isotonic NaCl. It was followed by a constant infusion of glycerol or glycerol and ethanol dissolved in isotonic NaCl and infused at a rate of 0.16 ml/min. The amount infused per minute was calculated from the body weight to be approximately equal to the maximum elimination rate at saturation. One hr was allowed for equilibration of glycerol and ethanol between blood and tissues, and blood samples were taken in heparinized tubes every 10—15 min.

By this procedure the time-concentration relationship was almost linear and could be followed for hours. Any change in elimination rate was indicated by a change in the slope of the line (Fig. 1). The amount of ethanol or glycerol retained in or disappearing from the solvent space of the organism per unit time was calculated from the slope of the line graphically best fitted to the experimental points. The elimination rate was then calculated by correcting the amount infused per minute by the amount retained or disappeared (Larsen 1963 a, b).

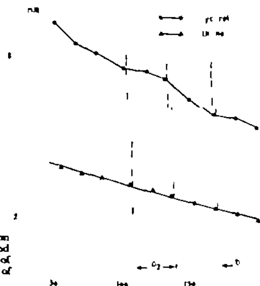


Fig. 1. The effect of oxygen breathing on the elimination rate of glycerol and ethanol. Weight 3 kg. Infusion rate of glycerol 94 μmoles/min. Infusion rate of ethanol 53 μmoles/min.

Administrat n of glycine

Glycine was given intrav nously in a dose of 0.5 g/kg body weight dissolved in 20 ml isotonic NaCl. In some experiments glycine was given as single injection i the course of 5 min in others it was given continuously in the course of 2—3 hrs.

Analytic l proc d r

Glycerol and ethanol were determined enzymatically as described in detail elsewhere (Larsen 1963 a, b) lactic acid ccording to Hohorst (1957) and free fatty acids as described by Dole and Meinertz (1960) Determination of arterial O and CO_2 tensions as w ll as pH were performed by means of micro-electrodes manufactured by Radiometer Copenhagen.

Results

Infusion of glycerol and ethanol

The depressive effect of oxygen breathing on the metabolism of glycerol was first observed in experiments on cats in which the oxygen uptake was determined during infusion of glycerol and ethanol The oxygen uptake was measured by connecting the cat to a closed system containing oxygen. It was then noticed that the elimination rate of glycerol was depressed during the period of oxygen breathing The mean elimination rate of glycerol in 7 expts. was 53 μmoles/kg/min (S D 8) when the cats were breathing air and 37 μmoles/kg/min (S D 3) when breathing oxygen. The elimination rate of ethanol was examined in 4 expts. and was found to be unchanged by oxygen breathing In 3 expts., the elimination rate was followed when air was substituted for oxygen and in these experiments the depressive effect of oxygen was found to be reversible A typical experiment is illustrated in Fig 1

In order to study this unexpected effect of oxygen the experiments were repeated, the depressive effect could not be reproduced. This series consisted of 8 expts 1) It was striking that the mean elimination rate in these experiment was low — 25 μmoles/kg min (S D 7) — as compared with the mean elimination rate in the first experiments. The reason for this difference in elimination rate is obscure

Infu i n of glyc ol ethan l and glycine

The results ind cated that th depressi e effect of oxygen might be dependent on a high elimination rate of glycerol. In previous unpublished experiments t was found that glycine was abl to increase the elimination rate of glycerol, and the effect of oxygen breathing wa therefore tested in a series of experiments in which the elimination rate of glycer l w incre sed by nf sion of glycine (Table I) Addition of glycine increased the elim nation rat of both glycerol and ethanol and breathing of oxygen now reduced th eli nation rat in 3 expts. whereas there w no effect in one experiment. Th ffect f o ygen on the elimination of ethanol was less pronounced, and in one experi e t th elimination remained unaffected

In a series of experiment l t n of the ureters was omitted. The amount of glycerol and ethanol lost thr gh th k dneys was measured in 2 separate expts in which a catheter was placed n th bladder and the urine collected at su table intervals and anal sed. Infus n f gl ine and breath ng of oxygen induced inconsistent and moderate changes he ex retion of glycerol and ethanol (Table II)

TABLE I The effect of glycine and glycine plus oxygen breathing on the elimination rate of glycerol nd ethanol

Cat no.	Substance	Basic elimination µmoles/kg/min	Elimination after infusion of glycine µmoles/kg/min	Elimination after infusion of glycine and during breathing of oxygen µmoles/kg/min
	Glycerol			
1		30	43	37
2		26	33	29
3		24	33	17
4		33	43	43
Mean		28	38	32
	Ethanol			
1		38	50	41
2		32	40	40
3		29	33	27
4		32	49	43
Mean		33	43	38

TABLE II The effect of glycine and glycine plus oxygen breathing on the urinary loss of glycerol and ethanol

Cat no.	Substance	Basic elimination µmoles/kg/min		Elimination after infusion of glycine µmoles/kg/min		Elimination after infusion of glycine nd during breathing of oxygen µmoles/kg/m	
		total	in urine	otal	in urine	otal	in urine
1	Gl cerol	40	13	54	12	47	12
	Ethanol	25	0 3	39	0 5	26	0 3
2	Glycerol	46	6	63	10	44	12

In experiments without ligation of the ureters (Table III) the eliminat on t of glycerol after dd tion of glycine was of the same order (56 µmoles kg min as the basic elimination in the preliminary experiments (53 µmoles kg mi n which the depres ive effect of oxygen was first observed. Apparentl the increase n elimination caused by glycine was almost completely inhibited by breathing of oxygen Fig

TABLE III The effect of glycine and glycine plus oxygen breathing on the elimination rate of glycerol and ethanol

Cat no.	Basic elimination µmoles/kg/min		Elimination after infusion of glycine µmoles/kg/ml		Elimination after infusion of glycine and during breathing of oxygen µmoles/kg min	
	Glycerol	Ethanol	Glycerol	Ethanol	Glycerol	Ethanol
1	24	27	44	39	22	31
2	33		54		31	
3	33		59		31	
4	36		52		40	
5	31		49		29	
6	48	31	81	45	47	30
7	39	28	59	37	41	31
8	42	30	62	47	47	43
9	45	35	68	57	53	52
10	21	21	37	40	31	36
Mean	35	29	56	44	37	37
S.D	9	5	13	7	10	9

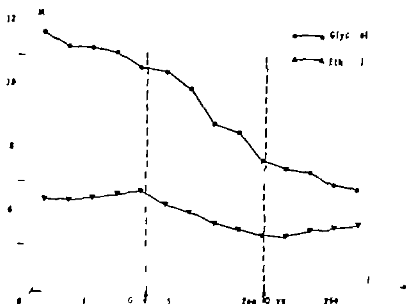


Fig. 2. The effect of glycine and oxygen breathing on the elimination rate of glycerol and ethanol. Cat no. 6, Table III. Weight 4 kg. Infusion rate of glycerol 140 µmoles/min. Infusion rate of ethanol 135 µmoles/min. At 136—138 min, 2 g glycine in 20 ml isotonic sodium chloride was infused. At 11 min, the cat starts breathing oxygen.

The effect on the elimination rate of ethanol was measured in some of the ex periments as listed in Table III The results are in agreement with the experiments listed in Table I

I fusi n f glycerol alone

The effect of oxygen breathing on the elimination rate of glycerol was studied in 9 expts. in which only glycerol was infused. In 8 expts. the mean elimination rate was 29 μmoles/kg/min (S.D 8) and there was no depressive effect of oxygen breathi ing On the contrary there was a slight increase in the elimination rate in ? expts. when the cat was exposed to oxygen. Only in one experiment did oxygen depress the elimination rate from 31 to 22 μmoles/kg/min. Breathing air increased the elimination rate to 38 μmoles/kg/min. In 3 expts. in which only glycerol was infused, the addition of glycine increased the elimination rate of glycerol in ? of the experiments. Breathing of oxygen increased the elimination rate in one experiment and was without any effect in the two other experiments. The mean basic elimina tion rate was 28 μmoles/kg/min.

Cha ges f fr e fatty acids and lact te concentration in plasma

The observed decrease in the elimination rate of glycerol might be caused by lipolysis, and therefore the concentration of free fatty acids was followed in 2 expts. In one of these the concentration of free fatty acids was almost constant, between 0.5—0.6 mM and in the other experiment there was a slight constant fall in concentra tion from 0 6—0.3 mM. In both experiments there was a definite effect of oxygen breathing on the elimination rate of glycerol.

In most of the experiments the concentration of lactate was not influenced by infusion of glycine or oxygen breathing However in a few experiments there was a definite but slight fall in concentration d ring oxygen breathing

Determ natio of blood gas tensions and pH

For technical reasons the blood-gas tensions and pH were not measured in ll ex periments. The available data on gas tensions are listed in Table IV It will be noticed that there is no significant difference in CO tension during air and oxygen breathing in the 3 groups. Th O_2 tension d ring breathing of air is the same in the 3 groups, and the O tension d ring breath ng of oxygen is also ndependent of the experimental conditions.

The pH of the blood was not hanged by the experimental proced res and was always within normal limits

Discussion

The effect of oxygen breathing may be explained by changes in the blood distribu tion in the li er but as the metabolism of glycerol and ethanol is influenced to dif ferent degrees by both glycine and oxygen, this pos bility is less probable Th ob-

TABLE IV The rterial tension of oxygen and carbon dioxide under various experimental conditions

Experiment	Breathing air		Breathing oxygen	
	PaO_2 mm Hg	PaCO mm Hg	PaO mm Hg	PaCO mm Hg
Infusion of gly erol	88±11	43±6	458±97	47±11
no effect of oxygen	=7	n=5	=7	=5
Infusion of gl cerol and ethanol	89±10	38±4	498±72	42±4
no effect of oxygen	n=8	n=7	n=8	=7
Infusion of glycerol and ethanol and glycine	83±13	40±7	505±70	43±7
ffect of oxygen	=14	=14	=14	=14

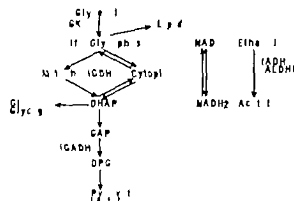


Fig 3. Metabolic pathw ys of glycerol and ethanol Abbreviations alpha-Glyc-phos alpha-glycerophosphate; DHAP dihydr oxy cetone phosphat GAP glyceralde hyde-3-phosphat ; DGP 1—3 diphospho glyceric acid (GK) glycerol kinase (GDH) glycerophosphat dehydrogenase (GADH) glyceraldehyde phosphate de hydrogenase (ADH) alcohol dehydrogenase; (ALDH) aldehyde dehydrogenase

served decrease in elimination rate of glycerol may also be caused by increased lipoly is lthough this explanation is not supported by the constant level of free fatty cid found in the experiments. In accordance with current knowledge of oxygen toxicity th demonstrated effect of oxygen may also be explained by inhibition of enzyme activity and accordingly the metabolic pathways and mutual influence of the substances involved will first be considered.

The metabolism f gly ol d tha ol The main pathways in the metabolism of glycerol and ethanol are outlined n F g 3. Normally about 70 per cent of the dihydroxyacetonephosphat DHAP formed is converted to gl cose and glycogen, and about 10 per cent is oxidized to lactate and carbon dioxide (Teng *et al* 1953, Nikkila and Ojala 1963 DHAP may however be reduced to alphaglycerophosphate by cytoplasmatic gl ero phosphate dehydrogenase (GDH) which in contrast to mitochondrial GDH i d pendent on diphosphopyridinenucleotide (NAD) (Baranowski 1963 Singer 1 63

It has been demonstrated in periments on humans and with rat-liver slices

(Lundsgaard 1946, Lundquist *et al.* 1965) that ethanol depresses the elimination rate of glycerol. On the other hand glycerol does not influence the elimination rate of ethanol. The effect was explained by the increase in NADH level caused by the oxidation of ethanol. This reduces DHAP and glycerophosphate accumulates with a corresponding decrease in ATP and increase in AMP (Thieden and Lundquist 1967). Apparently AMP inhibits the function of glycerokinase (GK) (Grunnet and Lundquist 1967). The depressive effect of ethanol could not be demonstrated in infusion experiments on cats (unpublished observations) nor was it observed in the present experiments.

The effect of glycine and oxygen breathing. Glycine is readily converted to pyruvate and in rat-liver slices pyruvate has been shown to increase the metabolism of glycerol (Teng *et al.* 1955). The effect of pyruvate or glycine on the elimination rate of glycerol may be explained by oxidation of $NADH_2$ with a corresponding increase in the activity of the NAD-dependent GDH and glyceraldehyde phosphate dehydrogenase (GADH) or by an increased production of ATP. The increase in the elimination rate of ethanol after addition of glycine may likewise be explained by an increase in the activity of the NAD-dependent enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH).

The depressive effect of oxygen breathing on the elimination rate of ethanol may be explained by an inhibition of ADH or ALDH. Both enzymes are dehydrogenases containing SH-groups, which makes them highly susceptible to oxygen poisoning (Dickens 1962) and yeast alcohol dehydrogenase is inhibited by oxygen at high pressures (Barron 1955). The depressive effect of oxygen breathing on the elimination rate of glycerol was only demonstrable in the presence of ethanol. In this situation ATP may be the rate limiting factor because of the fall in ATP concentration and the inhibition of glycerokinase (GK) by AMP. The effect of glycine may be explained by an increase in ATP production and the inhibitory effect of oxygen by a depression of the production of ATP. Sanders *et al.* (1966) exposed rats to pure oxygen at pressures of 1, 3 and 5 atm and found a decrease in ATP content in brain tissue at all three pressures. The ATP content of the liver and kidney was, however, only decreased at a pressure of 5 atm. The work of Horn, Haugaard and Haugaard (1965) and Horn and Haugaard (1966) has shown that the NAD-dependent enzyme GADH is inhibited by oxygen. Only a small part of glycerol is converted to carbon dioxide and lactate, but the inhibition of this enzyme may be of importance in the present experiments.

The mechanism by which increased oxygen tension may influence the tissue content of ATP is still obscure. The explanation may be an inhibition of SH-containing dehydrogenases with corresponding decrease in oxidative phosphorylation (Chance, Jamieson and Coles 1965, Jamieson and Chance 1966 and Sanders, Hall and Woodhall 1965). Sanders and Hall (1966) found no correlation between the decrease in ATP content of tissues exposed to oxygen at high pressures and the respiration and oxidative phosphorylation of these tissues when they were returned to normal ox

tension. This points against a reversibility of the effect of oxygen They found, how ever an irreversible decrease in the activity of succinic dehydrogenase which was correlated to the previous exposure pressure of oxygen.

In conclusion, the present experiment indicate that the demonstrated effect of oxygen on the metabolism of glycerol and ethanol may be explained in accordance with previous work in this field by assuming that oxygen at high pressures inhibits the function of specific dehydrogenases and the production of ATP

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Effect of pH and Temperature on the Uptake of Monoamines by Mouse Peritoneal Mast Cells in Vitro

By

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Abstract

JANSSON S. E. *Effect of pH and temperature on the uptake of monoamines by mouse peritoneal mast cells in vitro.* Acta physiol. scand. 1968 73 196—203.

The uptake of 5-hydroxytryptamine 5-hydroxytryptophan, noradrenaline and dopamine by normal mouse peritoneal mast cells was studied by means of the histochemical fluorescence method. It was found that 5-hydroxytryptamine uptake was significantly depressed by increasing pH from 7.0 to 8.9 and by lowering temperature to 20° C when the amine concentration in the incubation medium was 1 µg/ml but not when the concentration was 10 µg/ml. The 5-hydroxytryptophan uptake was to a high degree affected by similar pH and temperature alterations even when the concentration was as high as 200 µg/ml. This probably reflects a ... of the 5-hydroxytryptophan decarboxylase activity. Noradrenaline and dopamine was not significantly affected by pH or temperature alterations. These results indicate that normal mouse peritoneal mast cells take up 5-hydroxytryptamine by an active ... as well as by ... passive process and that the fluorescence observed after incubation with 5-hydroxytryptophan is due ... high degree to decarboxylation into 5-hydroxytryptamine. Noradrenaline and dopamine are apparently taken up by diffusion. Dopamine is taken up to some degree also by cells other than mast cells.

Reports on amine uptake of normal mast cells differ. Using radioactive isotopes Furano and Green (1964) stated that normal rat peritoneal mast cells take up exogenous histamine (H) and 5-hydroxytryptamine (5-HT) in vivo and in vitro but not dopamine (DA) nor noradrenaline (NA). Adams-Ray *et al.* (1966) presented evidence that mast cells of the hamster ear skin take up dihydroxyphenylalanine (D) and 5-hydroxytryptophan (5-HTP) in vivo but not DA, while Eränkö and Kaalo (1965) demonstrated that mesenteric mast cells of the mouse take up not only 5-HT and D but also DA and NA in vi... Eränkö and Jansson (1967) showed that mouse peritoneal mast cells take up D, DA, 5-HTP, 5-HT and NA also in vitro.

Day and Stockbridge (1964) found that ascites tumour mast cells take up ^{14}C-labelled 5-HT by an active process as well as by diffusion and that labelled H, NA and tryptamine were apparently taken up by diffusion. Cabut and Haegermark

(1966) showed that normal rat peritoneal mast cells take up H by diffusion. The present paper deals with the mechanisms of amine uptake by normal mouse peritoneal mast cells *in vitro*

Material and methods

About 160 expts. were made using some 50 adult albino mice of both sexes. The cells were obtained by rinsing the peritoneal cavity with Krebs salt solution, pH 7.0, supplemented with calcium chloride and glucose (Eränkö and Räsänen 1966). The cell suspension was resuspended in three or four 25 ml test tubes containing the Krebs salt solution t giv a final incubation volume of 10 ml and cell concentration of $4-7 \times 10^5$ cells/ml, both mast cells and leukocytes. The amines were added to the incubation medium in t different concentrations: 5-HTP 50 and 200 µg/ml, 5-HT 1 and 10 µg/ml, N and DA 10 and 50 µg/ml. The tubes were tilted in an almost horizontal position and they were incubated for 1 hr without agitation.

T study the effect of temperature on the amine uptake the incubations were made i a water bath both at 37 C and at room temperature. The effect f pH was estimated by adjusting the pH of the incubation medium to 7.0, 6.0 and 8.9 The adjustments to 6.0 and 7.0 ere made with phosphate buffers. As the Krebs salt solution became turbid at pH values abov 8.0 the incubations at 8.9 were made in Hanks salt solution without phenol red and buffered to this pH with a carbonate buffer The cells were also rinsed out with this sol tion

After 1 hr the incubations were interrupted by centrif ging the c ll down t about $100 \times g$ for 10 min. The cell sediment was then washed 4 times by resuspension i t the K ebs or Hanks solution without added amine and it wa collected by centrifugation. Smear prepar tions of the cell sediments were prepared by transferring droplets to the slides wit a glass rod

The preparations were air-dried and exposed to paraformaldehyde vapou under standa dized conditions (humidity 80 per cent, 1 hr t 80 C) according to Hamberger et al. (1965) to make the amines fluorescent (see Eränkö and Kauko 1965) After counting the fluoresc t cells in the fluorescence microscope the preparations were stained in 0.01 pe ce t aqueous toluidine blue and the mast cells in the same inspected area were counted The fluorescent percentage was obtained by expressing the number of fluorescent cells as percentage of the total number of stained mast cells.

Control incubations without amine were made t pH 7 0 tempera ure 37 C, simultaneou ly with the experiment incubations and the control preparations were tre ted wit formaldehyde vapour in the same reaction vessel as the experiment preparations. Cells from about 30 mice of different ages and both sexes were incubated without dded amine

The following amines were used 5-hyd oxy-DL-tryptophan (Sigma) 5-hydroxytryptamine creatinine sulphate (Fluka) 3 4-dihydroxyphenylethylamine HCl (Sigma) noradrenaline (L-arterenol-bitartrate) (Winthrop) All concentrations refer to the salt concent tions.

Results

The cells were in rather good condition after one hour s incubation and washings. They were somewhat distended but complete ruptures were of sparse occurrence After incubation t pH 6 0 or 8 9 mast cell granule clumping was somewh t mor common than at pH 7.0 A decreased metachromasia was also bserved (ll cell morphology and stainability was used as criteria for viability

The buffers proved effective in keeping the pH stable d ring the inc ba Checkings before and fter the ncubations showed no pH change gr t than on the meter read ngs.

Mast cells incubated without amines nd washed four times th re fter exhibited practically no fluorescence upon paraformaldehyde treatment (Fig 1 I with smear preparations, obtained without incubation or wash ng few mast cells showed an irregular extremely weak fluorescence mainly lt ca ed in the

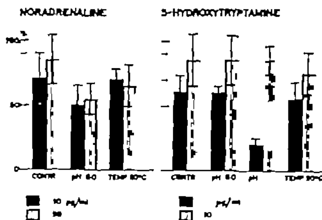


Fig. 9. Mast cell fluorescence after noradrenaline and 5-hydroxytryptamine incubation.

The scale gives the number of fluorescent cells as percentage of the total number of mast cells in the inspected area. The pH and temperature for the controls are 7.0 and 37° C respectively. Means and standard deviations.

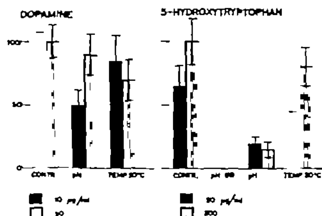


Fig. 10. Mast cell fluorescence after dopamine and 5-hydroxytryptophan incubation.

For explanations, see Fig. 9.

The results are presented in Fig. 9 and 10. As can be seen from Fig. 9 the percentage of fluorescent cells after incubation with N was 70 for 10 μg ml and 85 for 50 μg ml. No significant decrease in number of the fluorescent cells was achieved by pH or temperature lowering. The fluorescence intensity was also unchanged by these manipulations. *At a pH of 8.9 N and DA oxidized in a few minutes.* Therefore no incubations were made with these amines at this pH.

The 5-HT controls showed 60 and 85 per cent of fluorescent cells respectively. A lowering of pH from 7.0 t 6.0 h d nc effect on the number of fluorescent cells or fluorescence intensity. On th other hand a pH shift to 8.9 lowered the percentage of fluorescent cells to 15, the fluorescence intensity being extremely moderate when the amine concentration was 1 g ml, whil t no changes were seen t a concentration of 10 μg ml. A lowering of temperature from 37° C to 20° C did not affect the percentages of fluorescent lls of either amine concentration. The fluorescence intensity of the lower concentr t on was however *affected to a high degree*.

Fig. 10 shows the results of th DA and 5-HTP incubation. The percentage of fluorescent cells of the smaller DA oncentration is 90 and that of the higher is

marked as 100. A pH shift to 6.0 and a temperature decrease to 20° C ga e DA incubation results like tobe of N no depression of the percentage of fluorescent cells or fluorescence intensity was achieved.

5-HTP differed from the amines in that no percentage of fluorescent cells could be estimated when the pH of the incubation medium was 6.0 This was because the fluorescence intensity was so faint that no countings could be performed. As with 5-HT a pH shift to 8.9 depressed the percentage of fluorescent cells and fluorescence intensity to very low levels but differed from 5-HT in that the higher concentration was also affected. A further similarity with 5-HT was that a temperature decrease to 20° C depressed the percentage of fluorescent cells of the smaller concentration leaving the percentage of fluorescent cells and fluorescence intensity of the higher unaffected.

Discussion

That the mast cells fluorescence after formalin fixation was observed already in 1944 by Cramer and Simpson in precancerous formalin-fixed mouse skin. Fluorescence of normal mast cells of the rat was demonstrated by Lagunoff *et al* (1961) after treatment with formaldehyde vapour so as to render the amines fluorescent. However Fiori Donati *et al* (1962) stated that, after fixation in aqueous formalin, mast cells in the skin of the mouse do not show any fluorescence. Padawer (1966) reported that normal mouse peritoneal and mesenteric mast cells show a strong fluorescence after formaldehyde vapour treatment in smear or stretch preparations, while Eränkö and Kauko (1965) who also used formaldehyd vapour and stretch preparations, reported that normal mouse mesenteric mast cells do not exhibit any appreciable fluorescence. In the present study no significant fluorescence of mouse peritoneal mast cells was seen either It is difficult to explain these striking differences in results, although essentially the same methods were used. Dietary or strain differences in th binding of 5-HT by the mast cells could possibly be an explanation.

In the present study the 5-HT content, determined quantitatively with the spectrophotofluorometric method of Weissbach (1961) was found to be 0.2 pg cell. This alue is only about one fifth of the 5-HT concentration n peritoneal mast cells of the rat, reported by Ritzén to be 0.9 pg/cell, as determined using the method of Bogdanski *et al.* (1956) However a concentration of 0 2 pg/cell should be sufficiently high to be detected by the histochemical method

Since the number of fluorescent cells, observed after incubation w th 5-HTP 5-HT N or 10 µg/ml of DA was smaller than the number of mast cells, it seems that these amines were not taken up by cells other than mast cells assuming that all mast cells beha e in th same way On the other hand, after incubation with 50 µg/ml of DA, more fluorescent cells than mast cells were detected in 1 incubations out of 13 the mean percentage being 135, because DA was taken p not only by mast cells but also by small lymphocytes. Since the fluorescence disappeared even in those cells after sodium borohydride treatment and reappeared after renewed

formaldehyde treatment, it was due to an amine incorporation and not to autofluorescence (Corrodi, H. et al., 1964)

Adams-Ray *et al.* (1966) reported that in the mast cell of the hamster ear skin a yellow fluorescence was observed *only* after high dosages of 5-HTP and in combination with MAO inhibition. In the peritoneal mast cells of the mouse *in vitr* Eränkö and Jansson (1967) likewise observed that a comparatively high concentration of 5-HTP is needed to induce a fluorescence.

Adams-Ray *et al.* (1966) suggested that the fluorescence observed after 5-HTP is due to a decarboxylation of 5-HTP into 5-HT In the present work, a significant depression of the 5-HTP induced fluorescence was observed after incubation with 5-HTP at pH 6 0 and 8 9 instead of pH 7 0 This depression may possibly be due to depression of the 5-hydroxytryptophan decarboxylase activity whose optimum pH is 7.5 and which exhibits but a low activity at pH 6 0 or 8.9 (Lagunoff and Benditt 1959) 5-HTP exhibits only a weak fluorescence of its own (Jonsson 1967) and it seems therefore probable that the strong fluorescence of mast cells after 5-HTP incubation to a great extent is due to formation and storage of 5-HT

Day and Stockbridge (1964) found that lowering of the pH had little effect on the uptake of 5-HT by ascites tumor mast cells. However the uptake of 5-HT was much reduced when pH was increased to 8 9 as compared with the uptake at pH 7 0 when the amine concentration was 0 1 μg ml, while the uptake at pH 8.9 and 7.0 was about the same, when the amine concentration was 2.0 μg/ml. Temperature lowering always nhibited the amine uptake to a great extent, whatever amine concentration employed

The present results also showed a clear dependence of the uptake of 5-HT from amine concentrations on the pH and the temperature. It has been shown in an ier paper that normal mouse peritoneal mast cells take up 5-HT much more adily than other amines (Eränko and Jansson 1967) This observation also suggests an activ uptake mechanism.

Comparing the initial uptake against the amine concentration in the medium, Day and Stockbridge (1964) suggested that the uptake of N by ascites tumor mast cells was by diffusion Th present results also uggest that the uptake of N by normal peritoneal mast cells is pas i e DA behaved in the same way as N but t was taken up also by cell other than mast cells

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Capillary Permeability to and Extravascular Dynamics of Myoglobin, Albumin and Gammaglobulin in the Uvea

By

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Abstract

Bill, A. *Capillary permeability to and extravascular dynamics of myoglobin albumin and gammaglobulin in the uvea* Acta physiol. scand. 1968. 73 204—219.

The dynamics of myoglobin, albumin and gammaglobulin in the tissue fluids of the rabbit eye were studied in two-isotope experiments to elucidate the mechanisms for transcapillary movement of protein and protein drainage from the eye, the nature of the blood-aqueous barrier and the way in which extravascular protein may influence the formation of aqueous humor. The permeability of the blood vessels of the choroid and the ciliary processes to myoglobin was 10 to 15 times higher than that to albumin which was about twice that to gammaglobulin. In the tissue fluid of the ciliary processes the turnover rate constant for myoglobin was 25 cent/min, for albumin it was 3.9 per cent/min and for gammaglobulin 1.6 per cent/min. extravascular albumin spaces in the ciliary processes and the choroid were 158 µl/g and µg respectively. The extravascular albumin spaces were 68 and 57 per cent of the myoglobin spaces in the ciliary processes and the choroid, respectively. The extravascular gammaglobulin spaces were 87 98 and 117 per cent of the extravascular albumin spaces in the choroid, the ciliary processes and the rest of the anterior uvea respectively. The methods employed will give new information about the dynamics of the tissue fluids also in tissues with lymph vessels.

In a previous investigation (Bill 1964 a) it was found that in rabbits ten times more albumin passed out of the intraocular blood vessels than was drained with the aqueous humour. There is no system of lymph vessels in the eye and consequently it was not clear by which routes the main part of the extravascular protein passes out of the intraocular tissue. In a subsequent study (Bill 1964 b) it was found that protein injected into the suprachoroid left the eye by penetrating the scleral substance and by passing through perivascular spaces in the sclera. These routes could be presumed to drain protein from the choroid since there is no known barrier between the choroid and the suprachoroid. The protein drainage from the anterior uvea, that is the iris and the ciliary body, was less clear. The purpose of the present investigation was to study in more detail the dynamics of different proteins in the tissue fluids of the eye.

the ^{131}I-myoglobin concentration in the blood was maintained at steady level for 30 min and that of ^{125}I-myoglobin was maintained t steady level for 7 min. The procedure for maintaining these steady levels was the same as in the previous experiments, as also were the other procedures except that the eyes were not cooled before the dissections and that it was attempted to make the separation of the ciliary processes from the rest of the anterior uvea preparation more complete than in the other series performed. As consequence the time required for the dissection was longer Samples were taken also from the heart, skeletal muscle stomach wall and small intestine.

Albumin and gammaglobul

The animals were kept in a quiet room throughout the experiments. At the start 5 ml of a solution of labelled albumin and gammaglobulin were given intravenously In 30 min to 6 hr experiments additional doses were given every 15—60 min. In 24 hr experiments the doses after the first 10 hrs were given with 4—6 hr intervals. The animals had access to food and water

Small blood samples were taken and counted during the experiments which made it possible to adjust the additional doses so as to give an approximately steady blood concentration for each of the substances under study The animals were killed at different times after the first injection of labelled material by an intravenous injection of 60 mg pentobarbital sodium followed by 2—4 ml per cent KCl. The eyes were dissected in the same way as in the experiments with albumin and myoglobin. In these experiments samples were also taken from some other tissues.

Albumin and labell d d cells

^{51}Cr-labelled red cells and ^{131}I albumin were injected intravenously and the animals were killed 3 min or 5 hrs later after a blood sample had been taken. In the 5-hr experiments the ^{131}I-albumin oncentration of the blood was maintained t steady level as in the albumin-gammaglobulin experiments.

A small sample of iris tissu proper was taken from the small area of the iris that contains no visible ciliary processes. The ciliary processes were then dissected as in the experiments with albumin and myoglobin.

H matocrit

Hematocrits were determined by micro-capillary entrifuge 9000 g 10 min no correction trapped plasma was made

p'ocedure used to label the red cells was the same as in previous study (Bill 1964 a) labelled h man serum albumin was obtained from AB Atomenergi, Studsvik, Sweden. The labelling with ^{125}I and testing of horse myoglobin and human serum IgG-gammaglobulin (see Bill and Hellsing 1965) was kindly performed by Dr K. Hellsing. I all experiments the proteins were freed from iodine not bound to the proteins by gel filtration with Sephadex G 25

Assay f adioactivity

The radioactivity of the blood and tissue preparations was determined by two-channel gammaspectrometry (f B ll 1964 a, Bill and Hellsing 1965) All blood samples were counted long enough to make the cou ting error less than 2 per cent for each isotope. Most tissue samples were counted long enough to obtain counting error of less than 5 per cent.

Precipitation with 10 per cen trichloroacetic acid i the presence of some sodium ^{127}I iodide was used to determine whether significant amounts of radioactive iodine in the samples were due to radioactiv iodine not bound to protein.

Calculations nd definitions

The red cell olume within tissue sample was estimated by dividing the radioactivity due to ^{51}Cr (in cpm) in the sample by the corresponding radioactivity in cpm/µl of red cells in the blood. The plasma-equivalent ^{131}I-albumin space was calculated by dividing the amount of ^{131}I-albumi (in cpm) recovered in the tissue by the ^{131}I-albumi concentration (i cpm/µl) in plasma. The plasma-equivalent ^{125}I-myoglobin and ^{131}I-gammaglobulin spaces were calculated analogously I the following all spaces are plasma-equivalent spaces, turnover rate constants giv the rates of turnover as per cen of the pertinent steady stat spaces per minute

TABLE I. The pparent plasma equi alent ^{131}I-albumin and ^{125}I-myoglobin spaces after 0 nd 120 min of equilibration

	Plasma equivalent spaces µl/g			
	20 min		2 hr	
	myoglobin M±SEM	albumin M±SEM	myoglobin M±SEM	albumin M±SEM
aqueous humour	5.4±1.5	1 93±0.63	18.2±7.3	5.01±0 44
lens and vitreous humour	0.58±0.20	0.27±0.14	4.63±0 70	0.63±0.14
retina	14 0±1.9	8.8 ±1.2	19.3±2.8	9.5 ±1.3
cornea	5 4±0 7	1 79±0.31	20.9±2.8	3.39±0.80
ciliary processes	384±18	108±12	334±13	32±14
rest of anterior uvea	137±12	55±11	134±13	88±15
choroid	264±11	97.0±2 7	273±25	172±10
sclera	33.8±3.1	7 4±1 1	95±7	21 7±3 0

M = arithmetic mean
SEM = standard error of the mean

Turnover rates give values in plasma equivalent volumes per time per preparation, µl/min, or per gram tissue, µl/min/g. The total gammaglobulin space in tissue sample divided by the total albumin space in the sample determined at the same time is denoted gammaglobulin to albumin space ratio. The density of blood was assumed to be 1 05.

Results are presented as arithmetic means ± the standard error of the means.

Results

Five 20-min and five 2-hr experiments were performed with albumin and myoglobin. Table I summarizes the results.

Five experiments were performed with I myoglobin and ^{125}I-myoglobin. Fig 1 shows how the substances were injected and the resulting radioactivity of the blood in the coil. Table II shows the results.

With gammaglobulin and albumin 4—5 animals were killed 3 15 45 120 360 and 1440 min after the first protein injection.

Five 3-min and five 5-hr expts were performed with Cr labelled red cells and ^{131}I-albumin.

The experiments with albumin and myoglobin and those with myoglobin labelled with ^{131}I and ^{125}I are not quite comparabl with the others. The animals were under general anesthesia and the blood vessels to th kidney had been ligated. The general anesthesia was necessary to permit convenient continuous determination of th myoglobin concentration which tended to change rapidly In preliminary experiments without ligation of the kidney large amounts of ^{131}I not bound to myoglobin appeared in the blood. The reason for this phenomenon is not quite clear It is known, however that album that leaks out into the glomerular filtrate is to some extent

TABLE II. Total and extravascular myoglobin spaces per g tissue after 7 min and 30 min of equilibration

	Total myoglobin spaces µl/g		Extravascular myoglobin spaces µl/g	
	7 min space	30 min space	7 min space	30 min space
Ciliary processes	388 ±15	453 ±22	313	378
Rest of anterior uvea	75.5± 5.8	93.3± 8.3	48	67
Choroid	299 ±23	307 ±23	262	270
Sclera	33.9± 2.6	48.5± 2.6	23	40
Heart muscle	111 ± 3	161 ± 4	54	84
Skeletal muscle	12.8± 0.4	24.4± 1.0	6.5	18.9
Stomach wall	143 ± 6	203 ± 11	126	186
Wall of small intestine	79 ± 4	178 ± 8	55	154

reabsorbed and probably degraded in the proximal tubules (see *Maunsbach 1966*). Such a process with a smaller protein molecule as ^{125}I-myoglobin that easily passes out through the glomerular membranes could of course give a movement into the blood of ^{125}I not bound to protein. With the kidneys disconnected degradation of ^{125}I-myoglobin was no longer a problem.

The I-myoglobin I-albumin and ^{131}I gammaglobulin spaces of the ciliary processes

2 shows how labelled albumin and gammaglobulin accumulated in the ciliary ...cesses.

At each time interval there was a great scatter of data obtained so that if the

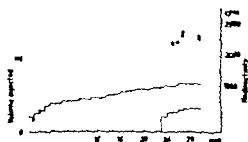


Fig. 1

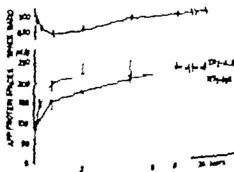


Fig. 2

Fig. 1. Myoglobin labelled w h I w given intravenously for 30 min in such w ... keep the concentration in he blood t steady lev l. During the last 7 min ...I-albumin also was given intravenously in he same w . The amount injected (accumulated) in ml is shown and also the resulting radioac ... of the blood.

Fig. 2. The pparent plasma equivalen spaces of ...I-albumin and ...I IgG-gammaglobulin t different times. The pparent IgG pac divided by the apparent albumin space is shown as space ratio. Vertical bars represent standard errors of means.

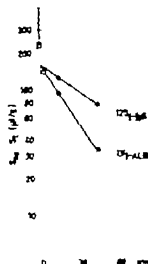


Fig. 5. Logarithmic plot against time of the difference between the steady state spaces (S_∞) for albumin and gammaglobulin and the spaces (S_t) observed at different times. The squares represent the steady state values.

^{131}I-albumin and ^{125}I-gammaglobulin spaces had been determined in different animals the significance of most differences would have been doubtful. The curve showing the ^{125}I gammaglobulin space divided by the ^{131}I-albumin space shows, however, that this ratio varied little from one animal to another at the same time intervals. The mean weight of the preparation was 8.87±0.21 mg.

Fig. 5 shows a semilog plot of the data for the ciliary processes. Like Fig. 1 this graph suggests that the labelled proteins became distributed within two compartments, one intravascular with a very high rate of turnover of albumin and gammaglobulin and one extravascular with low turnover rate. The turnover rates of albumin and gammaglobulin in the slow compartments are shown in Table III. Table I shows that the 20 min and 120 min myoglobin spaces in the uvea were very similar and Table II shows that this was also true for the 7 min and 30 min spaces determined simultaneously. In each preparation part of the myoglobin space was intravascular and was identical with the fast albumin and gammaglobulin spaces. In Table II the extravascular part of the myoglobin space has been calculated for each [illegible] subtracting the [illegible] part of the space from the total space observed at different times. O[illegible] pa[illegible]ng the 7 min and 30 min extravascular myoglobin spaces observed [illegible] th[illegible] same [illegible] it is seen that the 7 min space in the ciliary processes represented [illegible] average 83 per cent of the 30 min space. It is not possible [illegible] for myoglobin in the extravascular compartment [illegible] but on the assumption that myoglobin became distributed [illegible] vascular compartment in the ciliary processes an approximat[illegible] for the [illegible] rate constant could be estimated [illegible] Table III. In the [illegible] periments [illegible] albumin and myoglobin th[illegible] vascular myoglobin and albumin spaces were 259 µl/g and 177 µl/g respectively [illegible] the extravascular albumin space was 68 per cent of the myoglobin space. It might seem possible that some myoglobin was lost from the ciliary processes between the

TABLE III Characteristics of the protein spaces in the uvea

	Albumin			Gammaglobulin			Myoglobin		
	Ciliary processes	Rest of anterior uvea	Choroid	Ciliary processes	Rest of anterior uvea	Choroid	Ciliary processes	Rest of anterior uvea	Choroid
Apparent volume of fast space µl/g	75	26	37	75	26	37			
Apparent volume of slow space µl/g	138	38	163	135	68	144	378 (232)	67 (108)	270
Turnover rate constant, slow space, per cent/min	3.9	3.0	3.3	1.6	2.0	2.1	25	19	33
Turnover rate slow space µl/min/eye	0.033	0.076	0.120	0.022	0.059	0.06	0.76 (0.51)	0.62 (0.91)	1.94

of killing and the time of dissection. However when the myoglobin content of the ciliary processes in eyes dissected immediately after the killing was compared with that in eyes dissected about 5 min later there was no significant difference. It then also seems unlikely that any significant loss had occurred between the moment of killing and the dissection of the first eye.

Fig. 4 A shows how [131]I albumin and [125]I-gammaglobulin accumulated in the rest of the anterior uvea preparation. In this preparation the steady state gammaglobulin space was larger than the albumin space. The mean weight of the preparation was 45.6±1.2 mg. Steady state values for the spaces and the turnover rates were determined from semilogarithmic plots and the values for the extravascular myoglobin spaces shown in Table II were calculated in the same way as with the

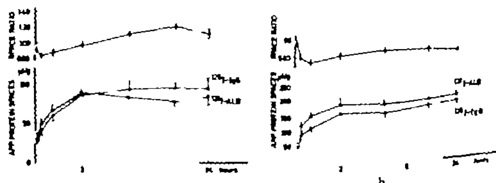


Fig. 4. The apparent plasma equivalent [131]I albumin and [125]I IgG-gammaglobulin spaces at different times and the space ratio obtained as in Fig. 2. A) gives the data for the anterior uvea preparation from which part of the ciliary processes had been removed. B) gives data for the choroid. Symbols as in Fig. 2.

ciliary processes. The 2 hr extravascular albumin space was 57 per cent of the 2 hr extravascular myoglobin space

A comparison between Table I and II shows that the myoglobin spaces in the ciliary processes and the rest of the uvea preparation were rather different in the two groups of animals investigated. The results indicate that the reason for the difference was the change in the dissection technique that made a smaller part of the ciliary processes with a high protein concentration to be included in the rest of the anterior uvea preparation in the experiments of Table II than in those of Table I.

The average weight of the ciliary processes in the experiments with more complete separation of them from the rest of the anterior uvea was not significantly different from that of the other groups indicating that due to the more timeconsuming dissections some fluid was lost from the preparation. If this fluid was aqueous humour adhering to the preparation or interstitial water remains unclear

In Table III to make the data comparable the myoglobin spaces of the 2 hr experiments have been included in brackets since these data were obtained with the same degree of separation of the ciliary processes from the rest of the anterior uvea preparation as the albumin and gammaglobulin data shown. To obtain also comparable approximate figures for the turnover rates of myoglobin in the different parts of the uvea th turnover rate constants determined were used to calculate the turnover rates shown in brackets.

Choroid

Fig. 4 B shows how labelled albumin and gammaglobulin accumulated in the choroid. The mean weight of the preparation was 21.8±1 0 mg Table I and II show the extravascular myoglobin spaces at different times and Table III shows the steady state values for the protein spaces and the turnover rates. There was no significant difference in myoglobin content between the choroids of the first and second eyes. The 2 hr extravascular albumin space was 57 per cent of the 2 hr extravascular myoglobin space.

Retina

In the retina there was no significant increase with time in the content of albumin or gammaglobulin in unanesthetized animals. The average ^{131}I-gammaglobulin space (including that in the blood vessels) in the 24 hr experiments was 7.8±0 87 µl/g, and for ^{125}I-gammaglobulin this figure was 6.3±0.3 µl/g. It was not possible to avoid contamination w th tissue fluid from the choroid during the preparation of the retina and in some cases traces of vitreous humour could not be separated from the retinal tissue. The mean weight of the preparation was 105±3 mg.

Sclera

Table I shows that in the sclera the 2-hr myoglobin space was much larger than the 20 min space. Therefore it remains unclear whether the steady state concentration

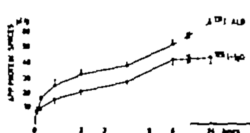


Fig. 5

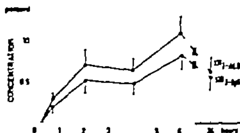


Fig. 6

Fig. 5. The pparent plasma equiv lent ^{131}I-albumin and ^{125}I IgG-gammaglobulin spaces in the sclera at different times. Symbols as in Fig. 2.

Fig. 6. The concentrations of labelled lbumin and IgG-gammaglobuli in the aqueous humour as per cent of the concentration in plasma. Symbols in Fig

of ^{125}I myoglobin had been reached even after ? hrs. Fig. 5 suggests that after 24 hrs of equilibration the steady state concentration for albumin and gammaglobulin had been reached. The mean weight of the sclera preparation was 507±5 mg.

Aque us hum u

Fig. 6 shows that in the aqueous humour the concentrations of the labelled proteins were very ariable. The gammaglobulin to albumin space ratio at 24 hrs was 0.96±0.10

rn a cit ous h nou and lens

Only very small amount of the labelled proteins were recovered in the avascular tissues of the eye after 24 hrs of equilibration the apparent albumin space of the itreous humour and the lens was 3.38±0.99 l g. The apparent gammaglobulin space was 2.65±0.87 l g. Contamination with tissue fluid from the uvea could not be wholly a oided. In th cornea the apparent I albumin space was 11.63±1.58 µl g and that for ^{125}I gammaglobulin 7.58±1.10 l g. The stead sate values are most probably higher than these values. The mean weight of the cornea preparation was 72.4±1.8 mg nd that of the itreous humour and lens preparation 1409±?7 mg

Other tissues

For the extraocular tissues n estigated the ariation in results was so considerable that turnover rates could not be determined. The 3-min and 24 hr labelled protein spaces were so different h wever that approximate alues for the extravascular protein spaces could be cal l t d. The data are shown n Table IV. It was assumed that the 3-min spaces were essen lly intra ascular and that the difference between the 3-min and 24-hr spaces ep esented the extra ascular prote spaces. The data for the ratio between the gamm globulin and albumin paces are summarized in Fig 7 although the results must be reg ded as only preliminary. To obtain approximate

TABLE IV The 3 min and 24 hr ¹³¹I-albumin and ¹³¹I-gammaglobulin spaces

	Spaces, µl/g				Average extravascular spaces, µl/g	
	3 min albumin	gammaglob.	24 hrs albumin	gammaglob.	albumin	gammaglob
	M±SEM	M±SEM	M±SEM	M±SEM	M±SEM	M±SEM
Heart muscle	83 ±3	77 ±7	147 ±6	131 ±7	62	54
Skeletal muscle	6.6 ±0.9	6.5 ±1.0	27.0±2.9	15.7±2.5	21	9
Wall of stomach	17.3±3.0	17.3±3.0	78.0±7.9	61.1±8.8	61	44
Small intestine	27.0±3.0	24.0±1.7	77.6±3.0	57.9±2.8	50	33

M = arithmetic mean
SEM = standard error of mean

values for the extravascular myoglobin spaces the 3-min albumin spaces were subtracted from the myoglobin spaces observed, Table II The 30-min myoglobin spaces should not be regarded as steady state spaces.

The blood content and the apparent extravascular plasma volume of the ciliary processes and of iris tissue proper

The red cell content of the ciliary processes after 3 min of equilibration corresponded to blood volume of 72.1±14.0 µl g tissue. In making this calculation it was assumed that the tissue hematocrit was the same as the large vein hematocrit (average 33 per cent) The apparent plasma content judged from the I-albumin concentration after 3 min of equilibration was 34.8±12.1 µl g higher than that expected from the calculated blood volume.

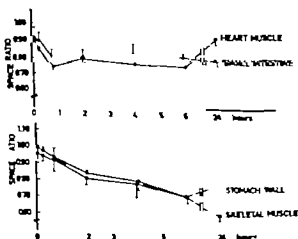


Fig 7 The ratio gammaglobulin space to albumin space in the small intestine stomach heart muscle and skeletal muscle Symbol as in Fig 2.

and 0.05 μl min for albumin and gammaglobulin respectively. When these rates are compared with those in Table III it is clear that they are of the same order of magnitude as the turnover rates in the uvea which supports the hypothesis that much of the plasma proteins leaving the uveal blood vessels passes out through the scleral substance and the perivascular spaces.

The drainage of myoglobin from the uvea

In rabbits the rate of aqueous formation is about 3.5 μl min (see Kinsey and Reddy 1964). In steady state the albumin and gammaglobulin concentrations were about 0.6 per cent of those in plasma. This means that albumin and gammaglobulin corresponding to the content of 0.0? μl plasma was drained by the aqueous humour per minute. In the ? hr experiments the myoglobin concentration in the aqueous humour was about 2 per cent of that in plasma. The steady state value may be somewhat higher but it may be concluded that the drainage of myoglobin with aqueous humour including any losses to the vitreous humour corresponded roughly to that in 0.10—0.15 μl plasma per min.

It can be calculated from the figures of Table III that the rate of turnover of extravascular myoglobin in the whole anterior uvea was 1.38 μl min, for albumin and gammaglobulin the figures were 0.13 μl min and 0.08 μl/min respectively. This means that in one min 1.38 μl myoglobin passed out of the blood vessels of the anterior uvea, while only 0.10—0.15 μl min was drained with the aqueous humour. The rest i.e. about 1.3 μl min had to leave the tissue by some other route. For albumin and gammaglobulin the corresponding figures were about 0.10 μl min and 0.05 μl min respectively. It cannot be decided in what way albumin and gammaglobulin passed out, probably both diffusion back into the blood vessels and drainage in bulk flow of tissue fluid from the tissue into the sclera contributed. With myoglobin the situation is clearer. The myoglobin concentration in the tissue fluid relative to that in plasma may have been somewhat higher than that of albumin and gammaglobulin but the similarity in the size of the spaces precludes great differences. This means that the rate of drainage of myoglobin with tissue fluid from the anterior uvea into the sclera cannot have been much higher than the maximum figure for this flow of albumin, say 0.10—0.15 μl min. In this figure may be included also any drainage due to diffusion into the sclera, since for anatomical reasons the rate of transport due to such diffusion can be presumed to be very small. Evidently the main amount of myoglobin corresponding to at least 1.10 μl min must have returned into the blood vessels. Since the return of myoglobin had to occur against a net flow of water the effective myoglobin concentration in the tissue fluid had to be higher than 80—85 per cent of that in plasma.

In the choroid also the turnover rate of myoglobin was much higher than that of albumin and gammaglobulin. For the same reason as in the ciliary processes the drainage of myoglobin with the tissue fluid can be presumed to have been about 10 per cent of the total or less and the effective myoglobin concentration in the tissue fluid was most probably similar to that in the ciliary processes. The fact that the

effective steady state myoglobin concentration in the tissue fluid was similar to tl m plasma makes it possible to use steady state myoglobin spaces as very importa links in determinations of effective albumin and gammaglobulin concentrations n *the ciliary processes and the choroid (Bill 1968)*

The formation of aqueous h m ur

As mentioned the rate of net water flow from the ciliary processes in rabb ts is probably about 3.5 μl/min. The weight of the ciliary processes preparation taken in the present *experiments was about 9 mg. If we assume that as much as 40 per cent* of the processes remained on the rest of the anterior uvea preparation, the total weight of the ciliary processes including minor parts of the non-secretory part of the ciliary body and traces of the zonule of Zinn and aqueous humour is about 15 mg. *This means that the ciliary processes have to produce an amount of aqueous humour* that corresponds to more than 25 per cent of their weight per m n. The rate of albumin turnover in the ciliary processes collected was 0.033 μl/min. If we ncl de also the parts of the ciliary body that were left on the anterior uvea preparation the outflow of albumin corresponds to about 0.1 μl/min, that of myoglobin to 1.3 μl min and that of gammaglobulin to 0.04 μl/min. The capillary wall thus restricts the movement of the normal plasma proteins out of the vessels to a ery large extent when it is compared with that of water (3.5 μl min) and small molecular sol tes. The large extravascular protein spaces of the ciliary processes which reflect high concentrations of the *proteins in the* tissue fluids (Bill 1968) show that none the less the more restrictive barrier to protein movement into the aqueous humour is represented by the ciliary epithelium.

In the above reasoning it was assumed that there is a balance between the rate of net filtration from th capillaries of the ciliary processes nd the rate of aqueous humour secretion. As pointed out previously it is possible that there is in fact a small flow of tissue fluid from the ciliary processes into the rest of the anterior u ea, that is, the rat of net filtration is a little higher than the rate of aqueous secretion.

It is of interest to consider in what w y the plasma proteins ma influence the movement of water in the ciliary processes. Two hypothetical models will be discussed, Fig 8. According to th first model sodium is transported actively into the spaces between the epithelial cells (see Cole 1966) chloride and w ter follow passively and there is competent barrier between the clefts and th stroma of the ciliary processes. Under such cond tions if the net outflow of water nd small molecular sol tes from the blood essels of the ciliary processes becomes somewhat lower than the rate of aqueous secretion the tissue pressure tends to f ll n the ciliary processes nd the processes shrink. Any drainage of tissue fluid conta ning protein into th rest of the anterior ea is reduced or may even become reversed temporarily. Th concentration of protei nd tissue polysaccarides in th interst tial tissue fl id rises and due to th red ed colloidosmotic transm ral pres ur difference and the increase in hydrostatic pressure d fferenc the net movement of w t out of the blood essels ncreases and soo new steady tate is tta ed t which

net movement of water out of the vessels equals the loss from the tissue due to secretion and movement of tissue fluid. If on the other hand secretion becomes somewhat lower than the rate of water outflow from the capillaries the concentration of protein and polysaccarides in the tissue fluid of the ciliary processes falls due to more protein drainage into the uvea and an increased amount of fluid in the ciliary processes. As a consequence the net movement of water and small molecular solutes out of the vessels is reduced.

Diamond (1965) has shown that in gall bladder preparations 40 mmoles per l of a nonpenetrating solute reduced the flow of water through the wall of the gall bladder by about 60 per cent. With the model considered here the conditions in the ciliary processes should be similar. Then moderate changes in extravascular protein concentration from a normal level of about 0.5 to say 0.7 mmole per l could be expected to have very small effects on the net flow of water through the ciliary epithelium.

According to the second model the barrier between the region into which sodium is pumped and the stroma of the ciliary processes permits movement of water chloride and sodium. Assume that sodium is pumped into the cleft between the epithelial cells and that water and chloride follow passively and that there is normally a net flow from the cleft into the posterior chamber and another net flow from the cleft into the stroma of the ciliary processes the former being due to a small hydrostatic pressure difference and the latter to osmotic forces. If the colloid osmotic pressure of the tissue fluid in the stroma is assumed to be 15 mm Hg this pressure will tend to attract water from the cleft. If the osmotic pressure of the fluid at the bottom of the cleft between the secreting cells is say 25 mm Hg higher than hat in deproteinated plasma and the permeability to sodium is such as to reduce fecti osmotic pressure difference to 13 mm Hg there will normally be a flow ter from the cleft into the stroma that is governed by an osmotic pressure head mm Hg. Assume that this makes 50 per cent of the fluid secreted return into the ciliary stroma, then it is obvious that a rise in colloid osmotic pressure from 15 to say 18 mm Hg will increase the return of fluid from the clefts into the stroma considerably and thereby reduce the flow into the posterior chamber. If the colloid osmotic pressure is reduced to say 12 mm Hg then obviously there will be a great increase in the te of flow from the cleft into the posterior chamber.

The real situat n is probably something between the two extreme models considered since at least n monkeys the rate of aqueous formation is influenced to a rather large extent by moderate changes in intraocular pressure (Bill 1967). Obviously the mechanism tlined abov may operate also in other secreting tissues.

The nature of a break-do f th blood-aq eous barrier

After some type of trauma in rabbit eyes (Sears 1960, Dyster Aas and Krakau 1964) and sometimes after administration of alpha-melanocyte stimulating hormone (Dyster Aas and Krakau 1963) the aqueous humour contains large amounts of protein. As mentioned above the normal rate of net filtration from the capillaries in

the ciliary processes is about 3.5 μl/min and the albumin of the filtrate corresponds to that in 0.1 μl plasma/min. The net filtrate thus has a protein concentration that is about 3 per cent of that in plasma or less. It is thus clear that a high protein concentration in the aqueous humour indicates not only that the barrier between the stroma of the ciliary processes and the aqueous humour is deranged. If the rate of formation of aqueous humour is of a normal order there must also be a breakdown of the barrier to protein movement through the capillary walls of the anterior uvea.

The retina

It is quite clear from the present and the previous results (Bill 1964 a) that if there is any extravascular volume in the rabbit retina into which albumin and gamma-globulin can pass from the blood vessels it is very small and that the turnover rate of protein in the extravascular parts of the rabbit retina is very low

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Acta physiol. scand 1968. 73. 220—225

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Relationship between Drug Induced Changes in Blood Pressure and Cerebral Oxygen Availability

By

S. M. Aquilonius, A. Sundwall and B. Winbladh

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Abstract

Aquilonius S. M. A. Sundwall and B. Winbladh. *R lationship betw n drug-in du d hange n blood p e u and c rebral oxygen availab l ty.* Act physiol. scand 1968 73 220—225

Under certai onditions proved possible to btain reproducible measures of hanges in ebral ox gen av labil ty aO_2, when using open-ended polarographic electrode I anaesthetized ts and rabbit rel ionship was found to exist between the blood pressure hanges produced by noradrenaline acet lcholine and hexamethonium, and their effect on cerebral aO_2. However the decrease in erebral aO_2 produced by acetylcholine and hexamethonium was normalized long befor he blood pressure was normal. In addition the incre se in aO_2 produced by ad enaline was als smaller than that produced by an equihypertensiv dose of radrenaline. This diff renc ma be due the fact tha adrenaline also increases the ebral oxygen onsumption. Potassium cy nide increased cerebral aO_2 w hou tsignificantly the blood pressu

This w rk has been ndertaken n order to test the usefulness of *oupl polarographic* electrodes f st d ing drug effects on cerebral oxygen availabil ty. Th term oxygen availabil t O w ntrod ed by Montgomery and Horwitz 1950. Although the d fficult of libr ting these lect odes has been considered a serious disad antage in circulation esea h Ingv r Lubbers and Siesjo 1960 Cater 1966 they might, under certain ond t ns pr v satisfactory for the study of local changes in oxygen a ailability. The present pape deal with the reproducibil ty of the method and the relationship between n labilit and drug induced hanges n rterial blood pressure.

Methods

The majority of the experimen erformed on rabbits anaesthetized ith uretha (1.4—1.6 g/kg) administered 3 exps made on cats anaesthetized ith intraperitoneal sodiumpentabarbitone (30—40 mg/k Dr gs ere injected through plastic annula into marginal ear ein or saphenous ei

Respiration was recorded, as pressure differences in the tracheal cannula, by means of pressure transducer (Model PT5 Grass Instrument Corporation) *Blood pressure* in the femoral artery was recorded with Statham Electromanometer

The polarographic electrodes were prepared from 30 µ platinum wire insulated in 100—150 µ glass capillary The tip of the electrode was polished transversally with fine abrasiv and the connecting wire soldered to the electrode. A one mm silver wire served as reference electrode.

The elect ode was polarized with constant oltage of —0.68 V from mercury cell. The reduction current was measured with a microamperemeter (Model 160 physiological gas analyser Beckman Instruments Inc) In the experiments with cats an lectrometer was used (Model 410 Keithly Instruments Inc.)

The function of all the electrodes was checked *i tro* in an ai -equilibrated, saline solution. The electrode response was very rapid and the final reading was obtained in less than te seconds. Electrodes were discarded, whose reduction current differed by more than 20 per cent from the mean or which drifted more than 5 per cent over 10-min period.

The lectrodes were positioned in the parietal cortex, using stereotaxic frame (David Kopf Instruments Inc) A hole was drilled through the parietal bone (4 mm posterior to the coronal suture and 3 mm lateral to the sagittal suture) the dura incised with hypodermic needle and the electrod carefully introduced to depth of 6 mm from the cranial vault, and fixed to the skull with dental cemen T avoid unphysiological brain pulsations the burr hole was filled w th dental cement The reference lectrode was implanted in the temporal or neck muscles. After insertion, the electrode was allowed to stabilize for t least 20 min, since the reduction current then eached plateau with maximum drift of 5 per cent. The plateau current aried considerably however between different experiments.

Standardization I order to be able to compare aO responses in different experiments, the drug-induced increases in aO_2 were expressed as fractions of the response to breathing mixture of oxygen and carbon dioxide (93.5 % and 6.5 %) for 30 sec The drug-induced de creases in aO_2 were standardized against the response to breathing nitrogen for 30 sec. The magnitude of the standard responses aried from one experiment to another, but were reproducible w hin 10 per cent during the course of 2—3 hrs. After the completion of the experiment the position of the tip of the electrode was erified in formalin-fixed, transverse sections.

I order to measure ariations in the oxygen content of the blood, repeated sample-oximetry was performed or pO_2 was continuously recorded by means of membrane covered polarographic lectrode I the former case, 0.5 ml of blood samples were drawn from the femoral artery and in the latter an oxygen microelectrode (Beckman Instruments Inc.) was inserted int he aorta through femoral rtery This electrode was calibrated in mm Hg oxygen partial pressure. The convectional sensitivity was less than 4 per cent and 95 per cent of the full response was obtained within 15 sec

Results

Following injection of noradrenaline (1—100 µg kg) the increase in arterial blood press re was accompanied by n increase in the cerebral aO (Fig 1) The increase in erebral O started either simultaneously with the increase in arterial blood pressure or with maximum delay of 5 sec. By increasing the dose of noradrenaline it was found that both the magnitude and duration of the increase in cerebral aO was pproximately correlated with the magnitude and duration of th blood pressure response. A plot of the maximum increase n O_2 against that in arterial blood pressure show direct relationship (Fig 2)

When the experiments were repeated with adrenaline (10—100 µg kg a simil relationsh p between cerebral O_2 and arterial blood pressure was observed but the slope differed from that obtained follow ng the administration of noradren line each experiment However when th results from different experiments were plotted together Fig the d fference was not statistically significant

When the increase in blood pressure w followed by transient decrease, this was also reflec ed the cerebral aO

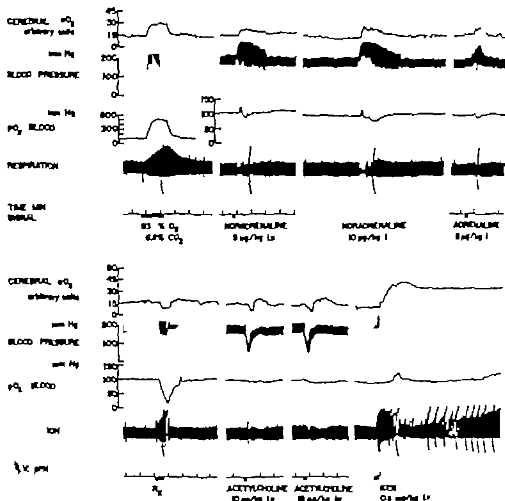


Fig. 1 Effect of noradrenaline adrenaline, acetylcholine and potassium cyanide on cerebral aO_2, blood pressure arterial oxygen tension and respiration in an anesthetized cat

Repeated sample-oximetry showed that there was no concomitant increase in arterial blood hemoglobin saturation with either adrenaline or noradrenaline In the 3 expts. with continuous recording of arterial oxygen tension, a transient fall in arterial pO_2 of 10—35 per cent occurred simultaneously with increase in blood pressure and cerebral aO_2 The decrease in arterial pO_2 lasted 15—180 sec and appeared to be associated with a change in respiration (Fig 1)

Following intravenous injection of acetylcholine (0.1—10 μg kg) the transient decrease in arterial blood pressure was accompanied by a simultaneous decrease in cerebral aO_2 (Fig 1) With larger doses of AcCh, when the fall in blood pressure was prolonged, the aO_2 was normalized before the blood pressure. A plot of the maximum fall in cerebral aO_2 against that in blood pressure suggests a curvilinear

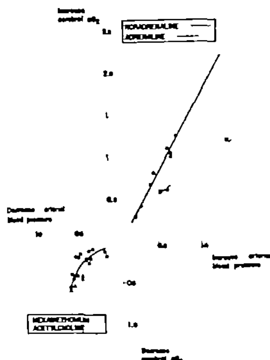


Fig 2. Relationship between changes in blood pressure and cerebral aO_2. Blood pressure changes expressed as fractions of initial values aO_2 changes expressed as fractions of responses to standard gas mixtures.

relationship (Fig 2) When the experiments were repeated with hexamethonium (10 mg/kg) and the maximal decrease in cerebral aO_2 was plotted against the maximum fall in blood pressure, the same relationship was obtained as with AcCh (Fig 2) However also in this case, the aO_2 returned to its plateau value long before normal blood pressure was restored (about 4 min and 20 min respectively) (Fig 3) Repeated sample-oximetry or continuous recording of blood pO_2 did not show any significant changes during the hypotensive period (Fig. 1) In about 20 per cent of the experiments a slight transient increase in cerebral aO_2 was seen immediately after its restoration.

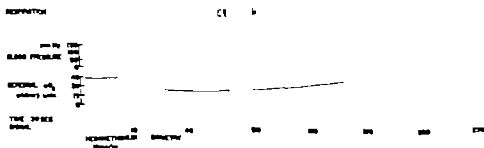


Fig 3 Effect of Hexamethonium on respiration, arterial blood pressure and cerebral aO_2 in the anaesthetized rabbit

Acta physiol. scand. 1968. 73 226—233

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Histochemical and Biochemical Observations on Cholinesterases of Cat s Tapeworm Taenia Taeniaformis[1]

By

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Abstract

ERÄNKÖ O K KOUVALAINEN M. MATTILA and S. TAKKI *Histochemical a d biochem al bservations n cholin steras of at tapeworm Taenia taeniaformis.* Acta phys ol. scand 1968 73 226—233

Cholinesterase activit of the tapeworm w studied histochemically w th the Koelle method, using acetylthiocholine and butyrylthiocholine as substrates, and biochemically w th the Warburg techinque using acetylcholine acetyl-β-methylcholine and butyrylcholine as substrates ne tetra-isopropylpyrophosphoramide iso-OMPA 1,5-bis(4-trimethylammonium-phenyl)-n-3-one diiodide BW 62 C 4 and 1,5-bis 4-allyldimethyl-ammonium-phen l pentan-3-diiodide BW 284 C 51 were used as inhibitors Both the histochemically and the bio-ypicall demonstrable tapeworm holinesterase activity was readily inhibited b low con ra ions of eserine but resistant the other inhibitors employed, wha ever substrate sed I this espect the tapeworm holinesterase activity markedly differed from that in the human serum the rat brain homogenat or the duodenum of the cat, which were used as referenc sources of holinesterase The histochemical holinesterase reactions obtained th acetylthiocholine and butyrylthiocholine with or withou iso-OMPA or BW 62 C 47 showed identical distributions, selectivel limited to nervous ganglia, nerv trunks and nerv fibres of the worm including those innervating he suckers. It is concluded that the tapeworm cholinesterase is distinc from mammalian acet lcholinest erase and non-specific holinesterase even if it is like acetylcholinesterase selectiv h neuronal enzyme and inhibited by excess substrat

PYLKKÖ (1956 wa the first t demonstrate acetylcholine and cholinesterase activity in the tapeworm *D phyllobothr m lat m* Paasonen and Vartiainen (1958) found that physostigmine increased the sensitivity to acetylcholine of isolated pieces of the cat tapeworm, *Taenia t f mis* In a manometric study acetylcholine or butyrylcholine as substrates it was observed by Mattila and Takki (1966) on the same worm that the cholinesterase activity was inhibited by therapeutic concentrations of several anthelmintics, suggesting that these may partly act by affecting the cholinergic transmission of the w rm

As far as we know the distribution of cholinesterases has not been studied in tapeworms with histochemical methods. However it is of considerable interest not only in view of the above observations but also as a general neurophysiological problem. The present paper deals with this subject and with further characterization of the cholinesterases in the tapeworm of the cat, supposedly *Taenia t eniaformis*

Methods

Histochemical method — Worms, free or attached to the duodenum of cats killed under brief ether anesthesia, served as material. Frozen sections were cut t 20 μ from fresh worms and worms fixed for 2—3 hrs in calcium-formalin mixture (Eränkö 1959) The sections were allowed to dry on the slide. Koelle (1951) copper thiocholine method was used for histochemical demonstration of cholinesterases, with acetylthiocholine (AThCh) or butyrylthiocholine (BuThCh) as substrate. The pH was 6 0.

Tetra-isopropylpyrophosphoramide (iso-OMPA) was used in the purpose t inhibit non-specific cholinesterase, 1,5-bis(4 trimethylammonium-phenyl)pentan-3-one diiodide (BW 62 C 47) to inhibit acetylcholinesterase, and eserine salicylate, to inhibit both enzymes. The inhibitors were incorporated both in the preincubation mixture in which the sections were incubated for 30 min before incubation with the substrate, and in the substrate mixture.

Quantitative method — Cholinesterase activity was measured using the Warburg manometric technique as described by Augustinsson (1948) Conical flasks of 5 5 ml olume were used, and the total olume of the reaction mixture was 0 4 ml. Augustinssons (1948) Ringer modification of the Ringer sol tion (100 ml of 0.9 % w/v N Cl 30 ml of 1.25 % $NaHCO_3$, 2 ml of 1.20 % KCl, and 2 ml of 1 76 % $CaCl_2 \times 6\ H_2O$) was used for making the homogenate and for diluting the drugs.

The worms were frozen and homogenized in mortar with 9 ml of salt solution for 1 g of worm. The final concentration of homogenized tapeworm in the reaction mixture was 1 20. Human serum (final dilution 1 40) and rat brain homogenate (1 40) were used as control preparations exhibiting almost selectively high activities of either non-specific or acetylcholinesterase, respectively

Acetylcholine hloride (ACh) acetyl-β-methylcholine chloride (MeCh) or butyrylcholine iodide (BuCh) served as substrates. The substrate was pipetted into the side bulb of the Warburg flasks. The cholinesterase inhibitors, pipetted with the homogenate into the main part of the essel, were iso-OMPA, 1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one di-iodide (BW 284 C 51) and eserine salicylate Cholinesterase activities were expressed as μl CO_2/g/hr Three or four separate determinations were made with each sample Control measurements were made using inhibitors and homogenate without substrate to heck non-specific formation of CO_2. Incubation wa carried out t pH 7 4

Results

Histochemical cha acterization of holi sterases. — An intense reaction was observed in th nervous ganglia, the nerve trunks and the nerv fibres of the worm with both AThCh and BuThCh These reactions were completely abolished by 10 M eserine indicating that cholinesterases were responsible. Fig 1 shows the reaction obtained in a fresh section with AThCh together w th iso-OMPA to inhibit non-specific cholinesterase presumably so as to demonstrate acetylchol nesterase selectively (see Eränkö 1959) The specimen is of a tapeworm ttached to th duodenum of a cat whose acti ty is also visible.

Fig demonstrates, in a nearby section of the same specimen, th reaction obtained with BuThCh used together with BW 62 C 47 in the purpose of hib ting acetylchol nesterase for selective demonstration of non-specific cholinest ase The distribution of the reaction is identical with that in Fig 1 A strong reaction of the same distribution wa obta ned i the worm also with AThCh. BuThCh iso-OMPA



Fig 1—3

Fig. 1 Distribution of cholinesterase activity towards acetylthiocholine and 10^{-6} M iso-OMPA of tapeworm attached to cat duodenum. Of the two suckers visible the right one is tangentially sectioned and shows the cholinesterase positive nerve net. Between the suckers an intensely reactive ganglion is visible. Note also the activity in the cat villi (upper left corner. Fresh section. ×35

Fig 2. Another section from the same preparation as that in Fig 1 but developed with butyrylthiocholine and 10^{-6} M BW 62 C 47. The distribution in the tapeworm is identical with that in Fig 1 but somewhat less extensive in the cat duodenum on the left. Fresh section. ×35

Fig 3 Same preparation as that in Fig 1 and 2 but showing the reaction obtained with acetylthiocholine butyrylthiocholine iso-OMPA and BW 62 C 47 all together. While the reaction in the host duodenum present in the upper part of the figure is entirely inhibited, the tapeworm cholinesterase is not affected. Fresh section. ×35



Fig 4—5

Fig. 4 Section of tapeworm fixed in formal-calcium. In this specimen, the rows with typical hooks can be seen to contain reactive fibres. Otherwise the distribution is similar to that in fresh specimens. Acetylthiocholine and 10 M iso-OMPA ×40.

Fig. 5. Another section of the worm shown in Fig 4 but with reaction obtained using butyrylthiocholine and 10 M BW 62 C 4. The distribution is identical ×40.



Fig 6—8

Fig 6 Part of mature section showing the genital apparatus Note the positiv cuticula nd the fibres apparently innervating the skin and the genital tube. F xation in formol-calcium. Acetylthiocholine and 10⁻⁶ M iso-OMPA × 45.
Fig. 7 Section simila to that in Fig 6 but developed in butyrylthiocholine and 10^{-5} M BW 62 C 47, with identical result.
Fig 8 Tangential section of the parenchymal muscle sheath showing the fine net of circular and longitudinal fibres with strongly reacti nodules in the sit of crossings F xation in calcium-formol. Acetylthiocholine and 10 M iso-OMPA. × 45

and BW 62 C 47 all together (Fig. 3) It should be noted that in this case the cholinesterase reaction of the host intestine isible in the same section, is completely negativ (*cf* with Fig. 1 and 2) indicating that the two inhibitors have together an eserine-like effect on the gut of the cat, although they are w thout effect on the rea tion n the worm.

Fig 4 is a section of a formalin-fixed worm, in this case a specimen fitted with a rown, incubated with AThCh and iso-OMPA. Fig. 5 is a nearly section of the same fixed worm after incubation with BuThCh and BW 62 C 47 again with an identical result Comparison of these figures with Fig. 1—3 clearly shows that fixation in f malin did not change the distribution or activity level of the reaction

Distribution of holin sterase activity — Cholinesterase-positive fine fibres hich originated from the strongly reactive dorsal and ventral ganglia and the commissures of the holdfast formed a dense network not only in the sucker but lw n he skin nd subcutaneous tissues outside the parenchymal muscle sheath (F g 1—5 Tissues inside the muscle sheath of the cranial segments were as a rule non-reactive In the more mature udal segments, a weak reaction was observed n the sperm duct, the testes nd the agina (Fig 6 and 7) E en in these segments the nervous reg

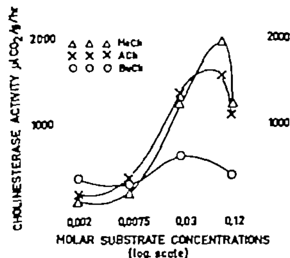


Fig. 9 Cholinesterase activity of tapeworm homogenate towards different substrates.

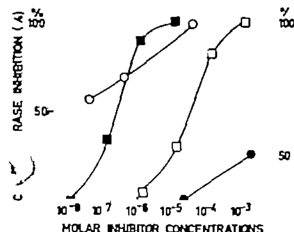


Fig. 10. Inhibition by eserine (open symbols) and Iso-OMPA (filled symbols) of the cholinesterase activity towards 0.12 M butyrylcholine of human serum (squares) or tapeworm homogenate (circles)

was distinctly more intense and made it possible to demonstrate fine fibres apparently innervating the genital tubes In the lower segments the cuticle showed an intense reaction, and beneath it a moderate reaction was seen in the skin (Fig 6 and 7) Fine nerve fibres extended to the skin and reached the positive cuticula. In tangential sections of the parenchymal muscle sheath, a network of fine nerves was seen. It was composed of both longitudinal and circular fibres (Fig. 8) Some of these fibres apparently innervated the muscle sheath, while others supplied the skin and underlying tissue with fine fibres

Manometric measurements. — Fig 9 presents cholinesterase activities in the tapeworm, as measured against varying concentration of ACh, MeCh or BuCh as substrates. The cholinesterase activity towards ACh and MeCh can be seen to be strongly dependent on the substrate concentration. The activity per g fresh weight was about a third of that of the rat brain towards the same substrates (3,500—4,500 µl CO_2/g hr) In the rat brain homogenate activity towards ACh was somewhat higher than

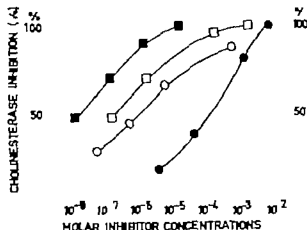


Fig. 11 Inhibition by eserine (open symbols) and BW 284 C 51 (full symbols) of cholinesterase towards 0.03 M acetyl-β-methylcholine of rat brain homogenate (squares) or tapeworm homogenate (circles)

that towards MeCh, but in the tapeworm both ACh and MeCh were equally hydrolyzed. The activity towards BuCh was very low in the tapeworm that of the human serum being more than 10 times as high (about 14000 µl CO_2/g hr) No clear dependence of the activity towards BuCh on the substrate concentration was noticed in the tapeworm.

Fig. 10 illustrates inhibition of the activity towards BuCh by eserine or by iso-OMPA in human serum and in the tapeworm homogenate. It shows that the tapeworm cholinesterase towards this substrat was more sensitive to eserine than th human serum cholinesterase. On the other hand, even high concentrations (10 M) of iso-OMPA were quite ineffective towards tapeworm cholinesterase although human serum cholinesterase was readily inhibited by much lower concentrations (10 M) of this inhibitor

Inhibition of activity towards MeCh by eserine or BW 284 C 51 is illustrated in Fig. 11 It can be seen that in the tapeworm BW 284 C 51 was much less potent in antagonizing the enzyme activity than eserine, whil the reverse pplied to the rat brain. Eserine almost equally inhib ted the activity towards MeCh of both th rat brain and the tapeworm homogenate.

Discussion

In the present study t was observed both w th histochemical techniques nd manometric measurements, that th characteristics of the cholinesterase act ty of the tapeworm distinctly differ from those of cholinesterases in most ther species (see Augustinsson 1963)

Firstly no difference was observed between the distributions of the histochemical reactions obtained w th AThCh nd iso-OMPA, on the one hand nd BuThCh nd BW 62 C 47 on the other In mammalian tissues, these two substrat -inhibitor combinations ha e been conclusively shown to demonstrate different enzymes, *i.e.* acetyl holinesterase and non-specific holinesterase respectively (*e.g.* Koelle 1963 Eränkö *et al.* 1964)

Secondly the tapeworm cholinesterase activity towards either of these histochemical substrates was resistant to simultaneous inhibition by iso-OMPA and BW 62 C 47 together. Since these inhibitors were together capable of inhibiting an equally intense reaction of the gut of the cat in the same section, technical errors d e to deteriorated inhibitors can be excluded.

Since the histochemical reactions with either AThCh or BuThCh were readily inhibited by 10^{-5} M eserine there seems to be little doubt that the reactions were due to cholinesterases even if these in the tapeworm cannot be divided into acetylcholinesterase and non-specific cholinesterase.

Manometric experiments also clearly showed that the cholinesterase activity of the tapeworm homogenate exhibited anomalous resistance to iso-OMPA and BW 284 C 51 which readily blocked under the same conditions the non-specific cholinesterase activity towards BuCh of human serum and the acetylcholinesterase activity towards MeCh of rat brain homogenate respectively.

On the other hand eserine proved a potent inhibitor also in manometric determinations of the tapeworm cholinesterase activity towards both BuCh and MeCh. Manometric and histochemical experiments th s furnished identical results concerning the effect of inhibitors.

The above observations are best explained by assuming that the tapeworm cholinesterase activity is due to an enzyme or enzymes whose substrate-inhibitor properties are different from those of mammalian tissues. The properties and the natural substrates of the tapeworm cholinesterase(s) remain to be further investigated. However inhibition due to increa ing the substrate concentration and the strictly neuronal ibution of the enzyme seem to indicate that the activity of the tapeworm cholin- activity whatever the specific substrate may be resembles more that of ace- lcholinesterase than that of non-specific cholinesterase.

From general neurophysiological point of view it is of considerable interest that the cholinesterase activity of the tapeworm is trictly neuronal, in the same y as it is in species hich are phylogenetically far from the tapeworm e.g. the mammals. This emphasizes the general importance of chol nesterases in nervous function. It is very likely that the presenc of the cholinesterases n the tapeworm is functionally associated with holine cetylase and acetylcholine whose especially great importance in the nervou function of man in ertebrat species has been pointed out by Hebb (1963).

It is of interest that ACh has been found to hav little effect on the spontaneous contractions of the tapeworm, unless cholinesterase activity was inhibited by adding eserine to the bath (Paasonen and Vartiainen 1958). This resistance to externally added ACh may have been d e to th intense cholinesterase activity as bserved in the present study which destroyed ACh before t reached the receptor sites.

O r observations suggest that great part of the nervous system of th tapeworm is cholinergic. This does, of course not exclude the presence of other transmitter substances, of which histochemical examination of cyclic monoamines should be done

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Dynamic Analysis of Muscle Spindle Endings in the Cat Using Length Changes of Different Length Time Relations

By

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Abstract

LENNERSTRAND G and U THODEN *Dynamic analysis of muscle spindle endings in the cat using length changes of different length time relations* Acta physiol. scand. 1968 *73* 234—250

Primary and secondary muscle spindle endings in the ankle extensors of the cat ere studied in response t length changes of ramp, triangular wa sine wa and parabolic wave form w th the intention of finding the most suitable input signal for an extensi dynamic analysis of the spindle during constant fusimotor input.

I dynamic tests the spindle endings were not found to obey linear length-response transfer functions no support for power function relation could be obtained. Sine wave analysis was therefore not considered suitable for the purpose of determining the transfer functions Atten- was directed towards the use of step changes of velocity nd of acceleration of length es. N evidence for specific sensitivity to acceleration was found in responses to 'para- length changes Responses interpreted as due to mechanical properties in the spindle efits resembling 'stiction were obtained when ramp length changes were applied These *cts were largely voided when recurring 'triangular length changes of constant velocity* were used It is concluded that the dynamic analysis might be adequately performed by subjecting the muscle spindles to periodically recurring length hanges of triangular wave form giving step hanges locity

The investigation, to be reported in this and subsequent papers, aims at a quantitative description of the static and dynamic properties of the muscle spindle endings. Such a description, to be presented in terms of a mathematical model, may derive either from the frequency response characteristics to sine wave *input or from* the transient response characteristics to step input.

Earlier studies on muscle spindle dynamics have been made mainly from impulse frequency responses to ramp shaped muscle stretches (giving step changes in velocity) both in the absence and the presence of fusimotor activation (Matthews 1962, 1963 a, Crowe and Matthews 1964 Brown, Crowe and Matthews 1965 Appelberg, Bessou and Laporte 1966, Brown Engberg and Matthews 1967) and the results

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have been expressed in terms of the dynamic index (Jansen and Matthews 1962, Crowe and Matthews 1964) In several respects these studies are not complete enough even for an approximate description of the static and dynamic properties of muscle spindle endings. In particular information is lacking on the following points 1) The different transients in the dynamic response to step changes in velocity have not been studied in detail. When the transients are known, a more complete description of the dynamic responses may be obtained than is inherent in the dynamic index 2) It has not been definitely established whether or not any of the transients can be regarded as a sign of a specific sensitivity to acceleration or higher order derivatives of length 3) The responses of the ending to muscle shortening, corresponding to the phase of contraction, has not yet been fully studied although from a functional point of view this phase may be considered as more important than the phase of passive muscle extension 4) In view of the increasing knowledge of fusimotor co-activation in natural muscle movements it is of importance to explore systematically the dynamic spindle response to variations in fusimotor input.

This paper concerns the selection of a length input signal from which the most significant information could be obtained for our subsequent dynamic analysis of muscle spindle endings at constant fusimotor activation. The starting point has been the suggestion of Brown and Stein (1966) that the muscle spindle endings may obey a power function input to output relation. In the case of a linear transfer function such as the power function relation, sine wave analysis would be the method of choice The power function hypothesis will therefore be considered first. The results of the present paper however do not lend support to this hypothesis. For reasons discussed by Matthews (1963 1964) and Crowe and Matthews (1964) other linear transfer functions seem unlikely Thus, sine wave inputs did not prove suitable for the further analysis. Recurrent changes of length of triangular wave form with step changes in velocity were found to be a better choice This input variable has therefore been employed in papers to follow Mathematical and physical models of the muscle spindle endings elaborated from the results of the physiological investigation, will be presented separately

Methods

Operative procedures. The experiments reported in this and following papers have been performed in a total of 45 cats weighing between 1 4 and 5 5 kg, anaesthetized with pentobarbital (Nembutal Abbott 35 mg—40 mg/kg b.w injected intraperitoneally) Supplementary doses of pentobarbital and 15 per cent glucose solution were injected as needed into the cannulated right femoral vein

Afferent activity from muscle spindles in the soleus and gastrocnemius lateralis muscles were recorded from dorsal root filaments. The surgical procedures comprised 1) laminectomy to expose the spinal segments L6 to S2, of which the ventral and dorsal roots were cut close to the spinal cord 2) peripheral denervation of the left hind limb except for the common nerve to the muscles under study 3) dissection of the muscles from surrounding tissue for at least two thirds of their entire length, 4) fixation of the left hind leg to a metal frame by screws and pins. Each muscle was attached to the myograph by a light metal chain sewed to its tendon near the muscle tissue. The spinal cord and the muscles were covered by warm mineral oil in pools made from skin flaps. Body temperature was maintained by means of a heating pad.

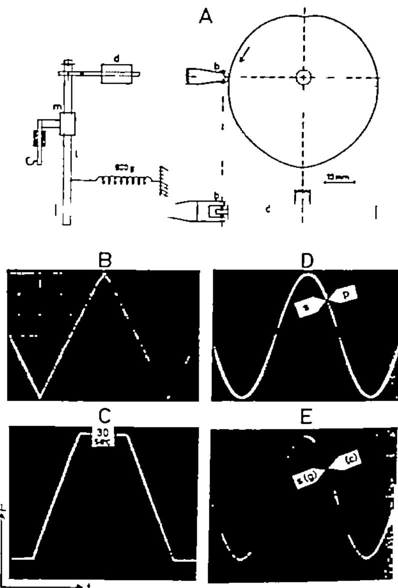


Fig 1 *A* show [illegible] schematically the arrangements [illegible] produce the length changes presented in *B* *C* *D* and *E* on [illegible] time scale *A* The [illegible] am [illegible] generate triangular length changes in *B* the ball-bearing *b* and [illegible] tachemen [illegible] draw both in top and in side [illegible] iew in the scale marked in the picture Other sections [illegible] show the arrangement to transmit the movement from the cam [illegible] the muscle are not [illegible] scale A coiled spring presses lever (*l*) and bar with ball-bearing against [illegible] am surfac [illegible] Fas ened to lever are the myograph (*m*) furnished with strain gauges ([illegible] to measure [illegible] ension and the core of the differential transformer (*d*) to record length. *B*. Recording [illegible] length [illegible] ariations of triangular wave form from the cam in *A*. *C*. Variations in length of ramp form Note A period of 30 sec constant length interposed between extension and relaxation *D* Length changes of parabolic wav [illegible] form (*p* from cam, compared to an electrically gene [illegible] ed sine wav [illegible] ( ) *E*. Curves of sinusoidal wav [illegible] from cam, ( ) and from [illegible] sine wav [illegible] ene [illegible] or [illegible] *g*) Scale on the ordinate identical in all length records. Time scale is in *C* differen [illegible] om tha [illegible] in *B* *D* and *E*.

Mechanical stimulation. The muscle was subjected to passive length changes (extensions and relaxations) of maximally 8 mm amplitude and of the length-time relations shown in Fig. 1. The length changes were generated by rotating cams of appropriate shapes driven at constant angular velocity. Figure 1 *A* shows the shape of the cam generating the changes of length with time referred to as 'triangular' (cf. Fig. 1 *B*). Figure 1 *A* also shows schematically the arrangements for transmitting the movements from the cam to the muscle. A bar with a small ball-bearing (*b*) of 4 mm outer diameter was spring-loaded against the surface of the rotating cam ( ). The bar in turn, acted on a lever (*l*) carrying a strain-gauge myograph (*m*) with a hook for the muscle. The spring pressing the bar with the ball-bearing against the cam resisted a pull on the myograph of 600 g, which was the upper limit of muscle tension during stretch. Variations in amplitude of muscle extensions were accomplished by altering the position of the myograph along the lever. The myograph for (isometric) recording of muscle tension consisted of a short, stiff, flat spring furnished with strain-gauges ( ). The length changes were monitored by means of a differential transformer (*d* in Fig. 1 *A*), the iron core of which was attached to the lever. The carrier frequency of the transformer was 20 kc/s.

The length-time relations which could be derived from the four cams were of approximately triangular (Fig. 1 *B*), ramp (Fig. 1 *C*), parabolic (Fig. 1 *D*) and sinusoidal (Fig. 1 *E*) shape. (The ramp length change had 30 sec constant length interposed between extension and relaxation and *vice versa*.) The appropriate shaping of the cams turned out to be difficult. The cams finally accepted for use showed only negligible deviations from the ideal shapes. The cam generating the sinusoidal movements had the largest error (7 per cent of the total amplitude.) By virtue of its position and shape in the sine wave period, the irregularity probably interfered very little with the measurements of response amplitude and phase angle. For the cams generating 'triangular' and 'ramp' movements the deviations from the ideal shapes were only 1—2 per cent. The corresponding effects in the impulse frequency responses were probably insignificant in comparison to the response variations obtained when identical stimuli were presented repeatedly. These variations amounted to 10—15 per cent around the mean and were not merely a sign of deterioration of the response with time. The errors of measurements from the recordings are included in the above figures.

For the parabolic cam no deviations from the ideal shape could be detected. The parabolic wave, which in Fig. 1 *D* is compared to an electrically generated sine wave, was actually composed of two parabolic curves of the general form $y = \pm \mp k$ each function expressing the induced length changes during one half-period of the cyclic movement. Step changes in acceleration resulted from the shift between the two parabolic curves during the movement in the manner shown in Fig. 9 *A*.

Maintained stretches of small amplitude for steady state measurements were accomplished by turning the cam by hand in steps. To generate recurring length changes the cams were driven by an electric (synchronous) motor (Norman Electric 1/10 h.p., 1500 rev/min) over a gear box which permitted the revolving speeds of the cams to be varied between 1/512 to 2 rev/sec in steps of one octave, i.e. a range of 11 octaves. Most of the results, however have been obtained within a range of 7 octaves between 1/32 rev/sec and 2 rev/sec. At the maximal amplitude and rate of length change (in the 'parabolic wave') the velocity reached 64 mm/sec which is about half the value of the maximal velocity observed in natural ankle joint movements of the cat (see Engberg 1964).

In order to avoid the effects of 'stray' vibrations from the motor and the gearbox, they were mounted separately from the recording table. This precaution proved sufficient since no effect on the discharge rate of spindle endings was seen by motor performance at different speeds when using a circular cam producing no periodic length changes.

Electrical stimulation. For selective stimulation of fusimotor fibres the rates of stimulation were 35, 70 and 200 pulses/sec (in a few cases 50, 100 and 150 pulses/sec). This range covers most of the naturally occurring discharge rates of fusimotor fibres.

Platinum wire stimulating electrodes were used. They were connected to the stimulator output through an isolation transformer. Rectangular pulses of 0.1 msec duration were mostly applied.

Recording. The length and tension monitoring devices have already been described. Both had linear input-output relations in the working ranges. No hysteresis was seen in length-tension plots when the muscle was replaced by a coiled spring of about the same compliance as the muscle. Zero extension was set at muscle lengths where the spindles discharged steadily and all slack of the muscle was taken up.

The activity in single spindle afferents were recorded from dorsal root filaments in a conventional way. The instantaneous impulse frequency was recorded by a device similar to that described by Matthews (1963 *b*). The frequency meter had a linear input-output relation in the range between 3 and 300 imp/sec with a relative accuracy better than 5 per cent. By means of a scaling circuit every second impulse could be rejected and the range of linearity

could thus be doubled (6—600 imp/sec). The impulse frequency calibrations given in the figures always refer to the true discharge rate of the spindle endings.

The correlates to length and tension of the muscle and t the impulse frequency signal were recorded photographically from two doubl beam cathode ray tubes. I addition to records on time scale diagrams of impulse frequency against muscle length or tension and of tension against length could be photographed from the screen of nother oscilloscope (T ktron 502). The majority of the illustrations are plots of spindle discharge frequency (f) gainst muscle length (ΔL) referred to as $f-\Delta L$ diagrams. The advantages of $f-\Delta L$ diagrams over the conventional records against time are firstly that direct comparison can be made between events occurring at the same muscle length but in opposite directions of movement and, secondly that plots from sev ral cy les of movements can be superimposed to giv average responses. F rthermore when muscle length changes of 'triangular' or ramp patterns are pplied, the plots have, i fact, linear time scale and the time course of the various phases in the re sponses can be determined.

Isolation f si gle f simotor f b es. The method used for isolating single fusimotor fibres to spindle endings followed roughly the methods outlined by Kuffler, Hunt and Quilliam (1951), Bessou nd Lapor (1962), M tthews (1962) and Crow and Matthews (1964). By the method -fibres were excluded. -fibres innerv ting the muscle out of study in the gastroc neumius-soleus muscle preparation were sometimes tolerated if the entral root filament, by its delicacy did not allow f rther subdi ision. Probably all fusimotor fibres of β-type, which by definition innervat both extrafusal and intrafusal muscle fibres (Adal and Baker 1965) were ho liminated.

In order to avoid fatigue of the intrafusal muscle fibres, fusimotor timulation was generally not performed when the period-time of the recurring length hanges exceeded 8 sec. For the same reason the experiments alway proceeded from the most rapid to the slowest extensions and to increasing rates of fusimotor f bre stimulation. Results obtained with rapidly declining fusimotor effects hav been excluded.

F actio al d t fication f pi dle affer t d efferent f br s. Muscle spindle afferents were identified in the conventional way by the pause in impulse firing during muscle twitch. Spindle fferents w th conduction locities abov 80 m/sec hav been categorized primary endings and those with conduction elocities below 60 m/sec as secondary endings (cf. M tthews 1964). The identification of 'dynamic' and 'static' fusimotor fibres have largely followed the general directions of Matthews (1962).

Results

S nusoidal hange f lengt

[Bro]wn and Stein (1966) have recently suggested that the input to output relation of the muscl pindle endings may be of power function type, i.e. a linear transfer function. The decay constant of the power function relation can be derived by using sinusoidal length changes as npu t signal as shown by Brown and Stein (1966). Thus, the decay constant, denoted as k, can be given either 1) by the slope of the plot in a log log diagram of the response amplitude (in imp/sec) against frequency of the sinusoidal input, or 2) by the constant phase lead of the response in ad ance of the sinusoidal input (90 k degrees). Both proced res have been employed in the present attempt to test th power function model on spindle endings.

In Fig. 2 are presented the amplitude changes and phase lead of the responses to sinusoidal changes of length of primary and secondary spindle endings in the absence and the presence of f umotor fibre stimulation. The steady state responses were subtracted from the over all response in the measurements of amplitude and phase angle, respectively. In log log plots of amplitude against sinus frequency an approximately linear relation was obtained for most endings. The k alues in Fig. 2 calculated from the amplitude changes, re given at each curve.

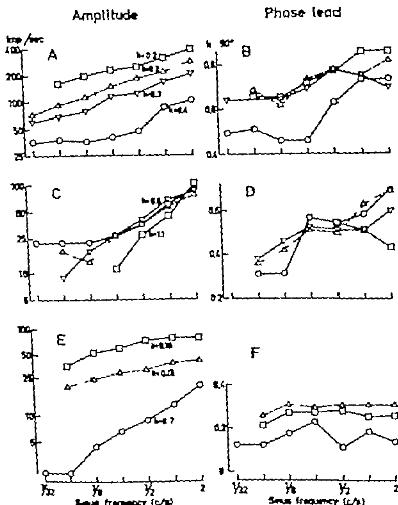


Fig. Some characteristics of spindle ending responses to length changes of sinusoidal wave form. Response amplitude ersus frequency of sinusoidal input show in A C and E. Phase lead of the response ersus frequency of he inpu illustrated in B D and F Response amplitude in impulses/sec on log scale phase lead in degrees on a linear scale and sinus input frequency in cps on log scale Gr phs in A and B from primary ending with and without dynamic fusimotor fibre stimulation C and D from another primary ending with and ithout static fusimotor fibre stimula ion E and F from secondary ending with and without static fusimotor fibre stimulation Symbol epresent no stimulation (O) stimulation (33 pulses/sec (▽) at 70/sec (△) and 00 sec □ The slopes of the curves in the response amplitude diagram, e. the alues of as m ked in the graphs.

Secondary endings of en exh bi ed a constant phase lead over the whole range of sinus frequencies pplied would be predicted by the power function h pothesis (Fig. 2 F) However f r most of the secondary endings tested the k alues obtained from the ampli ude sinu frequency plot differed considerably from those calculated from the phase lead Primary endings, on the other hand, generall showed an in-

crease in phase angle with increasing sinus frequency both in the absence and the presence of concurrent fusimotor fibre stimulation (Fig. 2 *B* and *D*). This is not in harmony with the power function hypothesis but is in agreement with the observations by Lippold, Redfearn and Vučo (1958) and by Stuart *et al.* (1965) (although the former authors did not identify the endings by their afferent conduction velocity). They found a constant phase lead only at sinus frequencies above 5—10 cps.

Moreover the differences in the *k* values obtained by the two methods were even more pronounced for the primary endings than for the secondaries but for both types of endings they were usually much too big to be explained by errors of measurement.

From these results it would seem that the power function model is unlikely at least in the frequency range used here. Nonlinear models are more likely (Matthews 1963, Crowe and Matthews 1964, Anderson and Lennerstrand 1966) and therefore further use of sine wave analysis seemed to be of little advantage for the elucidation of spindle dynamics.

2. *Length changes of constant velocity*

Having reached the above conclusion it seemed worth while to turn to expressions for the sensitivity to velocity and possibly to acceleration in order to accomplish the dynamic analysis. Such sensitivities can be determined from the response to input signals, when these signals are varied in a step-like or square wave manner. The input signals to generate steps in velocity have been the 'triangular' and the 'ramp' length change. It may be recalled that the extension and relaxation followed directly upon each other in the 'triangular' movements but that in the 'ramp' movements a period of 30 sec constant length was interposed.

At first it was necessary to determine the static or position sensitivity of each ending. This was expressed as the slope of the rectilinear plot of steady state impulse frequency (position response) against muscle extension (*cf.* Eldred, Granit and Merton 1953, for further references see Matthews 1964). The steady state values were measured 30 sec after the completion of a change in length. These values did not vary with the rate of change of length used to reach the constant length at which they were determined. In the record of Fig. 3 to show the measuring procedure the steady state curve is represented by the full line (Fig. 3 *B*). By subtracting in the individual case the steady state curve from the dynamically obtained impulse frequency curve, the response of the ending to the dynamic part of the length change, here called the 'dynamic response', can be acquired (see Fig. 3 *C*). Since the static stimulus to response relation is linear, it is assumed that the non-linear spindle responses depend mainly on its dynamic properties. It might be permissable therefore to subtract the static from the dynamically obtained response in such a non-linear system. The dynamic response may originate not only from the velocity sensitivity but may also arise from any possibly occurring specific sensitivity to acceleration and higher derivatives of length as well as from *e.g.* frictional forces (see below). At the beginning and end of each extension and relaxation a brief pulse of

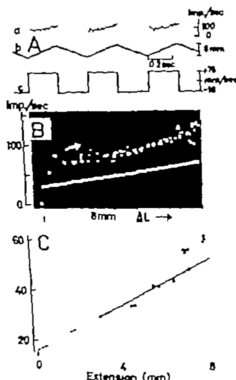


Fig. 3

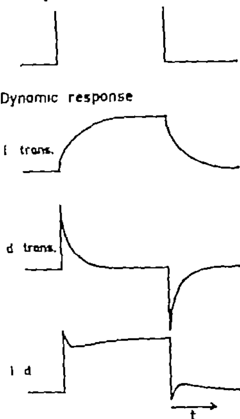


Fig. 4

Fig. 3 A Responses on a time scale (trace) of primary ending during three periods of recurring changes of 'triangular' movements (trace b) yielding step velocity changes (trace c). B The same three responses superimposed in an impulse frequency (f)—muscle length (ΔL) plot. Full line is straight line pproximation of the steady state curve. Arrow shows the point movement in time. Length increases to the right in the plot. C. The dynamic response of the ending to muscle stretch. Points obtained by subtracting the steady stat values from the alues in B. Full line fitted t the points by eye. Broken line is an extrapolation of f ll line towards zero extension. Note the time scale is linear also in B and C.

Fig. 4 Diagrams to show schematically on time scale the I- and d-transients in the response to a step change in elocity. The added response illustrated I the drawing be rs some resemblance to the dynamic responses of the secondary ending in Fig. 5 A and B

acceleration occurs, which possibly could give rise to short lasting specific responses to acceleration. Information on such and other transients in responses to tria gu lar and 'ramp movements are therefore of importance for a correct estimation of the velocity sensitivity

T ansient n th dynamic esponse If only a rectilinea relation between the rat of extension of the muscle and the dynamic response existed, the latter would tak the same time course as the applied change in velocity *I.e.* 'square wa e elocit

Ramp
A
Triang
C
B
D
Imp/sec
50
0
8 mm
ΔL →

Fig 5 $f-\Delta L$ diagrams of non-activated secondary ending to length changes of ramp (*A* and *B*) and triangular form (*C* and *D*) The velocity is approximately 2 mm/sec in *A* and *C* and 8 mm/sec in *B* and *D* Marking as in Fig 3 *B*

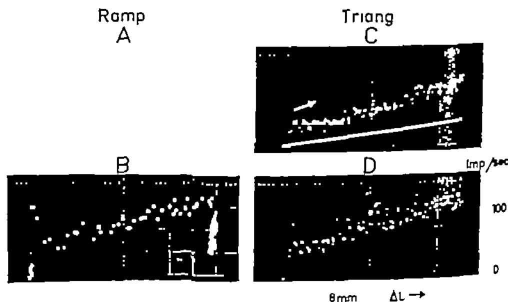


Fig 6. $f-\Delta L$ curves of non-activated primary ending to length changes of the same forms and velocities as in Fig 4 Markings as in Fig 3 *B*

steps in the case of 'triangular' and 'ramp' shaped movements. However by the occurrence of transients in the dynamic response this is not the case as can be seen in Fig. 3 *C*

Transients of quite different time courses were observed in the responses of both primary and secondary endings. They are schematically illustrated in Fig 4 One type of transient had a slow rise time and can be regarded as caused by mechanisms having some integrative effect on the dynamic response (see Fig. 3 *C* and 5 *C*) These transients will therefore be called 'integrative' or i-transients. Another type of transient showed a very rapid rise followed by a (quick) decay It was observed in the *f*—ΔL curves as a short lasting over shoot at initiation of the extension or the relaxation (Fig. 5 and 6) The mechanisms underlying these transients might be considered to be of derivative nature and consequently the transient will be called derivative or d transients. The summation of d and i-transients is shown schematically in Fig 4

By the existence of i-transients, the steepness of the *f*—ΔL curves will increase with increasing velocity of length change. This is seen in Fig 5 *C* and *D* showing the responses of a secondary ending to 'triangular' changes of length. The i-transients of primary endings were different from those of the secondary endings (see also Lennerstrand 1968) The variations in steepness of the *f*—Δ curves of primary endings in response to changes in velocity occurred within a range of much lower velocities than for secondary endings above 0.5 mm/sec there was no further increase in steepness with increasing velocity in primary endings (*cf* Fig 6 *C* and *D*) In secondary endings the change in steepness became significant at velocities above 1 mm/sec. For a more extensive discussion on these matters see Lennerstrand (1968)

Also during fusimotor activation the dynamic responses of spindle endings showed slow rises, the steepness of which increased with increasing velocity This indicates that i-transients in the dynamic responses are present also when the spindle is activated by its fusimotor nerves. The slow rises were seen during fusimotor activation of both the 'static' and the 'dynamic' type. The i-transients in the spindle activated by fusimotor fibre stimulation seemed to be similar to the i-transients of non-activated secondary endings the ranges of velocity in which the changes in steepness of the *f*—ΔL diagrams occurred were very much the same (Lennerstrand and Thoden, unpublished observations)

The part of the rapid d transients which are not masked by i-transients appeared as the 'initial burst response' of Jansen and Matthews (1962) and Matthews (1963) The study of d-transients and 'initial burst response' have led to the conclusion that different responses to length changes of the same constant velocity could be obtained, depending on whether 'ramp' extensions or 'triangular' movements were used The difference must be due to the periods of 30 sec constant length interposed between each period of extension or relaxation in the ramp pattern In the triangular pattern, extension was immediately followed by shortening and *vice versa*.

In primary endings the d-transients and the initial bursts were much more pronounced in response to the ramp input than in response to triangular movement

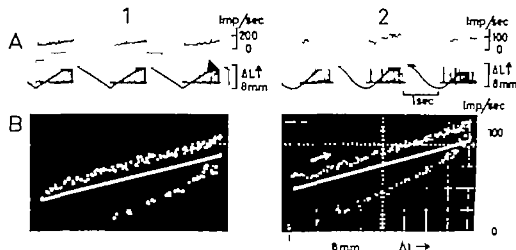


Fig 7 Initial burst responses in primary endings. *A* 'Initial bursts' observed in primary ending to both 'triangular' (*A* 1) and 'sinusoidal' length changes (*A* 2). Recordings from above instantaneous impulse frequency action potentials and length signal. Length increases upwards. Note, the difference in scale of impulse frequency in *A* 1 and in *A* 2. *B*. $f-\Delta L$ curves of primary ending to 8 mm/sec 'triangular' length cases. 'Initial burst' is absent in *B* 1 but appears in *B* 2 when the initial muscle extension is increased 2.5 mm. Marking as in Fig 3 *B*.

as can be seen by comparing Fig. 6 *A* and *B* with *C* and *D* The difference existed at all initial extensions of the movement. Shortening the time at constant length between extensions and relaxations in changes of length of a defined velocity resulted in a gradual decrease in size of the initial burst Initial bursts have been observed also in secondary endings but only to ramp changes of length (see Fig 1 and *B*) The d-transients were much reduced by fusimotor activation and initial bursts were completely absent in the responses to 'triangular' extensions but could sometimes be detected when ramp movements were used.

Are the d-transients related to acceleration? The d-transients which are so prominent in the response to ramp stretch in non-activated primary and secondary endings coincide roughly in time with a brief moment of acceleration. It would therefore not seem unlikely that the d-transients might signify a specific sensitivity to acceleration (Schäfer and Henatsch 1966, 1967) However the initial bursts were always found to be smaller in response to triangular' movements than in response to ramp stimulus (although the brief acceleration in both cases should be the same, i.e. infinite in theory) Further evidence has been obtained against the view that the 'initial burst is due to a specific sensitivity to acceleration. 1) initial bursts of similar magnitude have been recorded in response of primary endings to both 'triangular' and sinusoidal movements (Fig 7 *A* 1 and 2 see also the responses to 'parabolic extensions of the primary ending in Fig 9) although the maximum acceleration was much smaller in the latter type of input pattern than in the former 2) By decreasing the initial extension of the muscle, without altering the amplitude

or velocity of the movements, the initial burst of a primary ending could be abolished (Fig 7 *B* 1 and 2) (It should be noted that in both cases the tension at the lowest initial length was sufficient to prevent any slack in the main muscle) The d-transients and their manifest appearance as the initial burst therefore cannot be referred to as specific responses to acceleration. This is agreement with the conclusion of Jansen and Matthews (1962) The question whether any sensitivity to acceleration exists in spindles was subjected to further experimentation by step acceleration tests (see section 3)

Are all the v-transients related to velocity? It was shown in the preceeding section that initial burst responses or d transients could appear in the $f-\Delta L$ diagrams if the initial muscle length was increased while keeping the velocity constant (Fig 7 *B*) Therefore, besides being unrelated to acceleration, the d transients also seem to be velocity independent. It remains to be studied whether or not v-transients in the responses to step changes in velocity may also be influenced merely by changing the initial muscle length, in which case the transients would not be a true part of a specific velocity response. Such l-transients, if they exist, should then be excluded from the velocity response.

In secondary endings, both in the absence and in the presence of fusimotor activation, all the v-transients in the responses to steps in velocity at 'triangular' length changes were found to be independent of the absolute muscle length (see also Lennerstrand 1968) Initial burst responses were also absent under these circumstances.

However in some of the $f-\Delta L$ diagrams of non-activated primary endings at low velocity of 'triangular' change of length, l-transients were observed which were affected by changes in the initial muscle length. In these cases two types of v-tran-

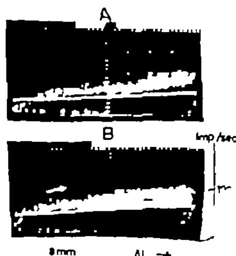


Fig 8 The two types of v-transients observed in the ... activated primary endings at low velocities. Rate of ... in both *A* and *B*. Initial muscle extension increase...

sients appeared. They can be seen in Fig. 8 where the two i transients are signified by the differences in slope of the initial and of the subsequent part of $f-\Delta L$ curves, the initial part beeing steeper than the rest of the diagram. When comparing Fig. 8 *A* and *B* it is evident that an increase in the initial muscle length without changing the velocity affected both the duration and the steepness of the initial part of the $f-\Delta L$ curves. On the other hand, in the later parts of the diagram, the slope of the dynamically obtained curve and its mean height above the steady state curve was maintained also at increased initial muscle length.

The conclusion would be that the i-transients appearing in the initial part of the $f-\Delta L$ diagram of non-activated primary endings is length dependent and cannot be regarded as a component of genuine velocity response. Moreover the physiological significance of these i transients, and also of the d transients for that matter is doubtful, since they disappear from the response as soon as the spindle endings are activated by fusimotor fibre stimulation even at a very low rate of stimulation. Fusimotor outflow through intact ventral roots would therefore in most cases prevent these length dependent i transients from appearing.

In the measurements of the velocity responses of the non-activated primary endings, the length dependent transients can be eliminated by extrapolating the slope of the later part of the $f-\Delta L$ diagram towards zero extension. This has been done in Fig. 3 *C*.

3. Length changes of constant acceleration

In order to subject the hypothesis on the existence of a specific sensitivity to acceleration (Schäfer and Henatsch 1966, 1967) to further tests, length changes of parabolic length-time relations have been employed whereby the acceleration changed in a square wave manner with no coinciding sudden variations in length or velocity (Fig. 9 *A*, cf. Methods). No change in the firing rate corresponding in time to the step change in acceleration was ever seen in the responses from secondary or from primary endings in the absence or the presence of fusimotor activation. Fig. 9 *A* serve to illustrate such experiments.

In Fig. 9 *B* the lack of a specific sensitivity to acceleration of the primary ending of Fig. 9 *A* has been certified in a more quantitative way. The response to position of this ending has been subtracted from the overall response and the result plotted on an expanded scale of impulse frequency. As shown in the Figure it was tested that the impulse frequency curve could be fully accounted for by the velocity response of the ending represented by the broken line and obtained from measurements in responses to triangular changes of length. No part was left for a response to acceleration and none appeared when larger steps in acceleration (up to 236 mm/sec^2) were applied to the non-activated endings or to endings activated by dynamic or static fusimotor fibre stimulation. These findings together with those concerning the origin of the 'initial burst' strongly indicate that primary and secondary endings do not possess any specific sensitivity to acceleration either in the absence or in the presence of fusimotor fibre activation.

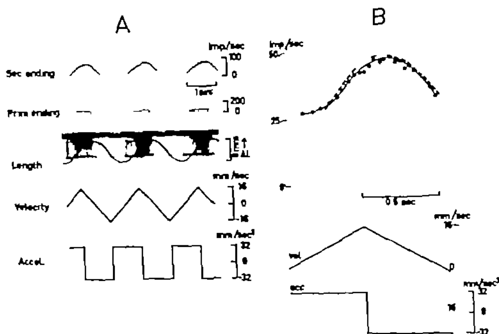


Fig. 9. A. Impulse frequency recordings on time scale of non-activated secondary and primary endings to 'parabolic' changes of length (the two upper traces). Action potentials from the primary ending recorded together with the length signal. Changes in velocity and acceleration during the 'parabolic' movements also shown. B. The response of the primary ending in A after subtraction of steady state values from over all response to 'parabolic' movements. Full line fitted to the points by eye. Broken line represents the velocity response calculated for the velocity changes shown in the middle trace. The negative step change in acceleration is shown in the lowest trace.

Discussion

The aim of the present paper was to find a suitable input signal which would yield as output the necessary information for a quantitative description of the spindle functions. The main result has thus been that changes of triangular length-time courses giving an input signal of square wave velocity steps can be adequately used for the further analysis of spindle ending dynamics both in the absence and in the presence of fusimotor activation.

Analysis by step changes in muscle length was omitted because this type of input is considered to be more easily distored by the viscous elements in the main muscle before reaching the spindle than other types of stimuli. In sinusoidal changes of length of about half the amplitude used here, such distortions have been reported to appear in the soleus muscle at frequencies above 2 cps (Roberts 1963, Rack 1966) and might thus have been present at the highest sinus frequency ap... In this study. The most rapid 'triangular' inputs have harmonics of a frequ...

2 cps and the responses to these inputs may therefore not be entirely reliable at a periodicity below 1 sec.

Results obtained with sine wave input signals are hard to interpret in case of nonlinear systems. At present there seems to be no conclusive evidence for the assumption of a linear transfer function in the relation between muscle spindle length and impulse frequency (Matthews 1963 *a*, 1964; Crowe and Matthews 1964; Andersson and Lennerstrand 1966). The hypothesis that linear transfer functions of power function type would apply generally to spindle endings could not be supported by the observations on the responses to sine wave input in the range of 1/32—2 cps. Although quite a few secondary endings responded as would be predicted by the power function model, the fact that primary endings did so very badly seems to be reason enough to leave the linear approach of sine wave analysis for the moment and turn to other methods whereby velocity and acceleration could be varied in a step like or square wave manner.

In the case of acceleration it was soon found out, by this method and by subsidiary pieces of evidence, that no specific sensitivity to acceleration is likely to exist in either primary or secondary endings. The arguments for this conclusion have already been given.

Thus, step changes in velocity would seem sufficient to yield the necessary information for the further analysis of spindle endings. Both ramp and triangular input signals have been used. In the responses to these two modes of generating step changes in velocity the existence of two different types of transients have been demonstrated. One type, the i-transients, was found to develop along time courses, which by their shape indicate underlying intergrative mechanisms, whereas the other type, the d-transients, suggested the presence of some derivative mechanisms. In contrast to the conclusion by Henatsch and Schäfer (1967) and Schäfer and Henatsch (1967) it was found that the d-transients and the initial burst cannot be regarded as signs of a specific sensitivity to acceleration. This is in agreement with the conclusions by Jansen and Matthews (1962). Our results fit well the view that the initial burst, which disappears during fusimotor activation, is due to some kind of dry friction in the intrafusal muscle elements (Jansen and Matthews 1962, Matthews 1963 *a*). The d-transients, which were so prominent in the responses to ramp length changes, diminished or even disappeared in 'triangular' movements. This implies another property of the proposed friction, the frictional force must be larger at initiation of a movement after a period of constant length than during continuous changes of length, a condition which by engineers is denoted as *stiction*. In order to as far as possible exclude stiction effects in the spindle ending responses, 'triangular' extensions were preferred to ramp inputs in order to generate step changes in velocity useful for the further analysis.

Some of the i-transients in the dynamic responses of non-activated primary endings were found not to be truly velocity dependent, since they could be influenced by changes in the initial muscle extension. These length dependent l-transients were observed in the initial part of the $f - \Delta L$ diagrams of non-activated primary endings

at low velocity The cause of these transients is obscure but they seem of very limited physiological significance, since they disappear when the endings are activated by their fusimotor fibres.

The length independent transients seem to be of much greater importance for the dynamic properties of the spindle endings. Their time course, for instance, is strongly influenced by the mode of fusimotor activation. This will be dealt with in greater detail in subsequent papers.

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Acta physiol. scand. 1968. 73 251—254

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Effect of Maternal and Fetal Hypophysectomy or Adrenalectomy on the Fetal Extra Adrenal Chromaffin Tissue of the Rat

By

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Abstract

LEMPINEN, M. and K. OJALA. *Effect of maternal and fetal hypophysectomy o adrenalectomy n th fetal extra-adrenal chromaffin tissue of the rat* Acta physiol scand. 1968. *73* 251—254

The present work was undertaken to study hormonal relationship between developing extra-adrenal chromaffin tissue and both fetal and maternal adrenal cortex. Maternal adrenalectomy performed during early or mid-pregnancy caused slight increase in the intensity of the chromaffin reaction of the full-term para-aortic body. Hydrocortisone substitution seemed to depress this effect. Maternal hypophysectomy performed in the late pregnancy caused complete disappearance of the chromaffin reaction of the extra-adrenal chromaffin cells of the full-term fetus while fetal hypophysectomy failed to produce any significant changes. It is suggested that maternal adrenocorticotropic hormone affects the chromaffin cells of the fetus during its late developmental stage.

Extra-adrenal chromaffin tissue of the rat is fully matured at birth, but without exogenously given corticosteroids it degenerates soon after birth (Lempinen 1964). It is also well established that cortical cells of the rat adrenal begin to function before the birth and that the hypophyseo-adrenocortical system operates t the end of the fetal period (for references, see Yakaitis and Wells 1956, Jost 1966). It is thus possible that development of the chromaffin cells is infl enced by the fetal hypophyseo-adrenocortical system. On the other hand the chromaffin reaction appears in the para aortic bodies already 15—16 days after copulation (Lempinen 1964) when fetal cortical cells probably are not yet completely functioning (see Yakaitis and Wells 1956 The question now arises whether maternal steroids, which are known to penetrat the placental barrier (Migeon t al 1961) or maternal ACTH provide sufficient hormonal stimulation for the differentiation of the fetal chromaffin cells.

Material and methods

I rats of he Sprague Dawley strain, pregnancies were timed by mating. The females were either in the late stage I or in the stage II of the estrous cy le as determined from aginal smears, when the females were placed i the cage with males for —4 hrs.

Maternal adrenalectomy was performed on the morning of the 5th day and on the 14th day after copulation. The mother was anesthetized with ether and adrenals were removed through an abdominal incision. NaCl was added into their drinking water but otherwise they were fed on standard diet. Three rats in both groups completed pregnancy. One animal in each group was daily injected with 25 mg of hydrocortisone acetat (Hydro Andreson, Organon) between the 14th and 19th days of pregnancy.

Transauricular hypophysectomy (see Falconi and Rossi 1964) was performed on the 17th (1 rat survived) or on the 16th (2 rats survived) day of pregnancy. A specially modified hypodermic needle connected to a water suction-pump was introduced in the left auditory canal. The needle kept at a proper angle was then pushed forward following the bony canal until the medial wall of the periotic capsule was reached. With a light pressure the bone was perforated and pituitary gland sucked through the needle. The completeness of hypophysectomy was determined by microscopic examinations of serial sections from the ablated tissue and of the pituitary region. Postoperatively NaCl and glucose were added into drinking water but no hormonal substitution was given. The experiments were terminated by killing the surviving mothers on the 22nd day of pregnancy.

Fetal hypophysectomy was performed on the 16th or 17th day after copulation by decapitation in utero. The method was a modification of the one described by Domm and Leroy (1951). Only one or two fetuses in each horn were operated, the others serving as controls. Four decapitated fetuses were found alive at Caesarean section on the 22nd day of pregnancy. The others were either dead or completely resorbed. As a control subtotal decapitation (encephalectomy) was performed, leaving the hypophyseal region intact (2 fetuses survived).

After weighting the fetuses and the newborn rats were killed by bleeding and the retroperitoneal tissue block containing both kidneys and adrenals were fixed in bichromate-formol solution for demonstrating the chromaffin tissue (for details, see Lempinen 1964). Some specimens were also prepared for histological studies.

Results

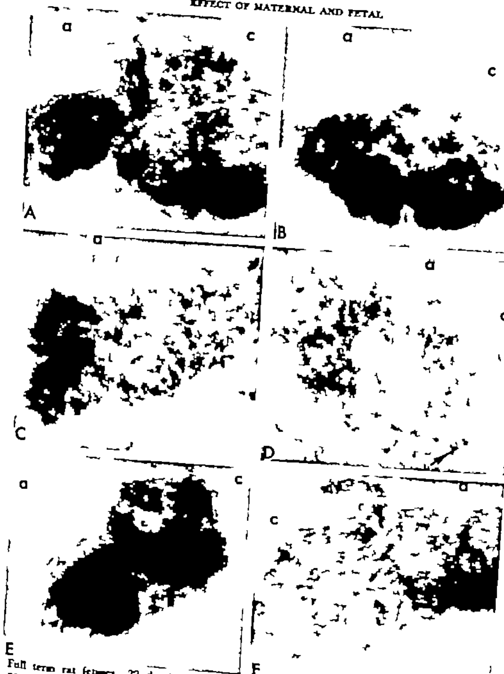
Chromaffin tissue

In all surviving experimental animals the localization and histological picture of the extra-adrenal chromaffin tissue was similar to that of untreated newborn. The bulk of the chromaffin tissue outside the adrenals was in the main para-aortic body as in normal newborn only a few small groups of chromaffin cells were seen in the vicinity of the abdominal sympathetic ganglia. Occasionally some faintly chromaffin cells were also noticed inside these ganglia.

Fig. 1 A illustrates the chromaffin reaction in the main para-aortic body of the control. Maternal adrenalectomy regularly caused a clear increase in the chromaffinity of the body (B); in the para-aortic body of the offspring of hydrocortisone-treated adrenalectomized rats (C) there was no significant difference in the chromaffinity from the untreated controls. Maternal hypophysectomy caused complete disappearance of the chromaffin reaction in the extra-adrenal chromaffin cells (D). Fetal hypophysectomy and encephalectomy had no significant effect on the chromaffinity of the extra-adrenal chromaffin cells (E and F).

Discussion

It has been suggested that in adrenalectomized pregnant rats some of the maternal ACTH crosses the placenta (Knobil and Briggs 1954) and that the placenta acts only as a partial barrier to ACTH (Yakaitis and Wells 1956). It has also been shown that in pregnant hypophysectomized rats there is no extra pituitary ACTH mobilized in response to stress (Schapiro and Geller 1964). On the other hand, there is also evidence that maternal adrenalectomy has no effect upon the blood corticosterone levels in fetal and newborn rats (Milkovic and Milkovic 1961).



Full term rat fetuses (22 days). Transverse sections through the middle part of the main para-aortic body. Bichromate-formol fixation. Frozen section ×200 — aorta, [illegible] cava. A Untreated control. The body gives uniform chromaffin reaction. B Maternal adrenalectomy. Operation performed on the 5th day after copulation. Intensity of the chromaffin reaction is stronger than that seen in the untreated body. C Hydrocortisone substitution. Maternal adrenalectomy as in Fig. B. The body gives uniform reaction. Intensity is the same as seen in untreated control. D Maternal hypophysectomy performed on the 16th day after copulation. The body is uniformly non-chromaffin. Small darker spots (arrows) are blood in capillaries. E Fetal hypophysectomy (on the 17th day after copulation). The body gives uniform chromaffin reaction. The intensity does not differ significantly from that [illegible] ly in the body. F [illegible] from the same litter as that in Fig. E. [illegible] chromaffin reaction is [illegible] seen normally at this age.

The increase in the chromaffinity of the extra-adrenal chromaffin cells due to maternal adrenalectomy and the reverse effect caused by maternal hypophysectomy observed in the present study suggest that maternal ACTH either directly or through the fetal adrenal cortex affects these cells.

In the present study fetal hypophysectomy did not affect the chromaffinity of the extra adrenal chromaffin cells (both adrenaline and noradrenaline content) Jost and Roffi (1958) observed that there was a significant decrease in the pressor activity of the adrenals (mainly due to adrenaline) in decapitated rat fetuses and an increase in corticosterone treated ones. Eränkö *et al* (1967) have shown that in newborn rats injected with hydrocortisone *in utero* the adrenaline content of the extra-adrenal chromaffin tissue much increases as compared with normal untreated controls, in which only noradrenaline can be detected (Lempinen 1966, Eränkö *et al.* 1966, Roffi and Margolis 1966) Lempinen (1964) has also shown that ACTH in massive doses prevents the postnatal degeneration of the extra-adrenal chromaffin cells.

These observations together with the present suggest that, although ACTH probably affect the chromaffin cells through the adrenal cortex, it may also have a direct effect on the function of the chromaffin cells which probably differs from that caused by the adreno-cortical hormones.

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Pentose Shunt Enzymes in the Crustacean Stretch Receptor Neuron after Impulse Activity

By

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Giacobini and Grasso (1966) studied the levels of glycolytic intermediates, pyridine nucleotides and phosphate compounds in the isolated slowly adapting stretch receptor neuron of the crayfish (SRN) after prolonged stimulation *et* The results strongly suggested that the breakdown of glycogen is involved in the mechanism maintaining impulse activity in this neuron. In order to study other energy producing pathways during impulse activity a series of experiments was initiated to measure pentose shunt (hexose-monophosphate shunt) enzymes and substrates.

In the present communication the activity of the two oxidative pentose shunt enzymes, glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6GPDH) is reported

The SRNs used in this investigation were dissected from abdominal segments of crayfish (Astacus fluviatilis) during April and May The technique we used for dissecting and handling the preparation was essentially the same as described by Giacobini and Grasso (1966) except that during registration of the 30 000 impulses no mineral oil was applied over the preparation and the axon was raised above the sol tion. Control (at rest) and SRNs stimulated by stretch from the same dorsal segment of each animal were assayed simultaneously The dry weight of the cells was measured with quartz fibre balance.

Optimal substrat concentrations and pH optima for G6PDH and 6PGDH were determined using homogenates of the abdominal ganglia chain.

Single SRN preparations were incubated t 30° C for 1 hour in 3 μl of an incubation mixture of the following composition

G6PDH 0.1 M 2-amino-2 methyl-1.3-propanediol (AMP) buffer pH 9.1 5 mM glucose 6-phosphate, 0.5 mM NADP 10 mM $MgCl_2$ 1 mM EDTA, 0.05 % bovine plasma albumin (BPA)

6PGDH 0.1 M AMP buffer pH 9.1 1.5 mM 6-phosphogluconate 0.8 mM NADP 2 mM $MgCl_2$, 0.5 mM EDTA. 0.05 % BPA

The tubes were placed in an icebath nd the rea tion was stopped w th 22 μl 0.03 N NaOH. Excess NADP was destroyed by heating fo 15 min t 60° C

After appropriate dil tions with 0.03 N NaOH th extracts were enzymatically cycled (Lowry *et al* 1961) and fluorimetrically measured.

When the enzyme activ ty was tested in several aliquots belonging t the same extract (i.e. same cell) the values showed a very high reprod bility (± 5)

The enzyme activities were expressed in μmoles of substrat converted per milligram dry weight of tissue during 1 hr incubation t 30 C.

The 90 % increase in G6PDH activity after 30,000 impulses (Fig 1)

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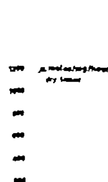


Fig 1 Fig 2

Fig. 1 The effect of 30,000 impulses on G6PDH in SRN
o — enzyme activity of control cell.
• — enzyme activity of stimulated cell.
O the abscissa are eported different experiments including the stimulated cell with its control. The cells on the same vertical belong to the same animal. Means and standard errors are indicated on the right.
Fig. 2. The effect of 30,000 impulses on 6PGDH in SRN
Same symbols as Fig. 1
δ = 222 µmoles/mg/hr dry tissue.

significant (Student t t = 5 5 $p<0.001$) Although a small increase (15 %) of 6PGDH activity could be observed (Fig 2) no significant difference was present (Student t t = 0.45 $p>0.5$) even if we applied statistical tests to the differences of the paired observations (Rank symmetry test of Wilcoxon $p>0.1$ Student t on red differences t = 0 43 $p>0.5$) On the other hand, levels of stimulation as i as 100 000 impulses did not produce any increase of 6PGDH activity

The approximate 2-fold increase in G6PDH activity after stimulation does not cessarily imply *de novo* synthesis of the enzyme but could possibly be explained by an unmasking of the active site of the enzyme molecule.

Whether or not this increase has some consequences for the utilization of glucose 6-phosphate through the oxidative part of the pentose shunt during impulse activity cannot be answered although the augmented glucose 6-phosphate level in SRN (Giacobini and Grasso 1966) makes this possibility very likely The variation in pentose shunt int rmediates which is reported elsewhere (Giacobini 1968) provides additional support to the role of this metabolic pathway in maintaining impulse activity in the SRN

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Acta physiol. scand. 1968 73 257—263

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The Effect of Exercise on the Development of Collateral Circulation after Experimental Occlusion of the Femoral Artery in the Cat

By

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Abstract

Sanne, H. and R. Siverrsson. *The effect of exercise on th development of collateral circulation after experimental occlusion of the femoral artery in th cat* Acta physiol. scand. 1968. 73 25 —263.

Flow resistance in the collateral vessels passing an occlusion of the femoral rtery was deter mined 5 weeks after the occlusion in 24 cats kept in small cages and 21 cats trained daily on treadmill. The collateral resistance as determined at maximal vasodilatation to reveal bether structural outgrowth had taken place Despite the occurrence of pronounced "spontaneous outgrowth of the collateral essels it could be shown that collateral resistance was 30 per cent less in the trained group This difference was statistically highly significant $(0.01 > p > 0.001)$ On the other hand, flow resistance in the ascular bed, distal to the oc lu sion, did not change after the arterial occlusion and was not affected by training

In recent years many authors ha advocated physical training as a therapeutic aid n patients with myocardial infarction or with intermittent claudication (Ratschow 1959 Varnauskas t al 1966 Sanne and Selander 1967) An increased walking tolerance is observed in patients suffering from intermittent claudication who exer cise regularly for a number of months (Larsen and Lassen 1966, Hedlund and Porjé 1964 The mechanism affecting this ncreased tolerance is, however unknown.

The possibility of nfluencing the growth of ollateral vessels is of great interest Eckstein 1957 found signs of increased development of collateral essels when dogs with experimentally occluded coronary arteries, were trained

The aim of the present study was to examine the effect of physical exercise on the development of collateral essels when the main artery of a limb was occluded ex perimentally in cats The present results were reported briefly i surveys given by Folkow 1967) and Thulesius (1967 at an International Symposium 1 St dies of Peripheral Circulation, held in Copenhagen 1966.

Methods

45 cats with an average body weight of 4 kg were used. The cats to be trained were chosen before the arterial occlusion on the basis of their willingness to run.

In all the cats the superficial and deep femoral arteries were ligated on one side during brief ether anesthesia. After one week, the animals to be trained started daily physical exercise on a treadmill. They ran five days a week, two hours a day, at a speed of approximately two kilometers per hour. The speed and duration of exercise gradually increased. The cats received their food during the exercise period in order to stimulate running. The control animals were kept in small cages measuring 70×90 cm throughout the course of the experiment.

Five weeks after the arterial occlusion, an acute experiment was performed. The cat was anesthetized with a combination of chloralose and urethane 50 and 100 mg per kg b.w. respectively and a tracheal cannula was inserted. The intestines were extirpated and the blood flow to the hind paws was eliminated by means of tight ligatures around the ankles. Body temperature was kept constant by an electrical heating pad.

After heparinization, the femoral vein on the occluded side was cannulated just proximal to the adductor channel of the thigh and the venous outflow (Q) was measured by means of an optical drop chamber-ordinate writer unit recording on a kymograph. The systemic blood pressure (P_{system}) was measured in the carotid artery and the pressure at the distal end of the arterial collateral vessels (P_{local}) was judged by measurement of the pressure in the femoral artery peripheral to the ligature (Fig. 1). For these pressure measurements mercury manometers were used.

Measurements of the collateral flow resistance were performed during maximal vasodilatation to eliminate errors inherent in changes of vascular tone. As a rule this was induced by means of an acetylcholine infusion into the central end of the inferior mesenteric artery. The dose was increased in a stepwise fashion from 2 to 36 µg/min. The skeletal muscles were often activated as well. For this purpose the sciatic nerve was divided close to its origin and its distal end was stimulated at a rate of 40 imp/sec for 1 min with intensities high enough to activate all the somatomotor fibres but not the postganglionic vasoconstrictor fibres. Blood flow was recorded both during and after this period of intense muscle activation.

In both the trained and untrained animals, the contralateral previously intact hindlimb was used to determine the minimal resistance of the collateral vessels and the resistance of the local vasculator bed beyond the collaterals, after an acute arterial occlusion. The same method was used as described above.

In both limbs the minimal resistance of the collateral vessels ($PRU_{collateral}$) and of the local vascular bed (PRU_{local}) could be calculated in the following way (see also Thulesius ...)

$$PRU_{collateral} = \frac{P\ system - P\ local}{Q} \quad \left(\frac{mm\ Hg \times min \times 100\ g}{ml}\right)$$

$$PRU_{local} = \frac{P\ local}{Q} \quad \left(\frac{mm\ Hg \times min \times 100\ g}{ml}\right)$$

After the experiment, the muscles of the calf were weighed. All blood flow values were related to 100 g of this muscle portion in order to compensate for difference in cat size and for possible muscle hypertrophy caused by the training. It should be observed, however that the

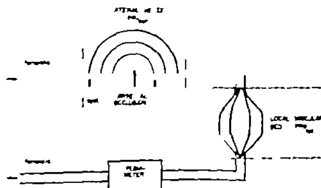


Fig. 1. Schematic drawing of the experimental situation.

Table I

	Trained animals			Untrained animals			Significance of difference
	Mean	SD	n	Mean	SD	n	(Students t-test)
Immediately after occlusion							
Collateral resistance (PRU_{coll})	3.52	1.14	13	4.13	1.16	15	$p > 0.1$
Local resistance (PRU_{local})	1.24	0.44	12	1.19	0.56	13	$p > 0.1$
Five weeks after occlusion							
PRU_{coll}	0.99	0.30	21	1.37	0.49	24	$0.01 > p > 0.001$
PRU_{local}	1.15	0.44	20	1.23	0.43	24	$p > 0.1$
Blood flow at minimal PRU_{coll} (ml/100 g × min)	47	14.8	1	37	10.7	24	$p > 0.1$
$P_{systemic}$–P_{local} at minimal PRU_{coll} (mm Hg)	41.2	10.6	21	50.9	15.5	4	$0.02 > p > 0.01$

preparation also included muscle portion proximal to the knee point, also small portion of bone and cutaneous tissue. For such reasons the present resistance values should not be directly compared with those given by other investigators.

The data were analysed according to Student's t-test and rank-sum test (Dixon and Massey 1957).

Results

Spontaneous development of collateral vessels

The spontaneous growth of the collateral vessels in the limb proved to be very good. During the 5 weeks after the arterial occlusion, the collateral resistance during maximal vasodilatation decreased almost exactly threefold in the untrained animals (Table I and Fig. 3). This implies that the "normal" stimuli for collateral development, whatever their nature, are very efficient.

Effect of training on collateral and local flow resistances

Immediately after the *acute* arterial occlusion in the previously intact limb, the flow resistance of the collaterals at maximal vasodilatation was 15 per cent less in the trained cats (3.52 compared with 4.13, see Table I and Fig. 3). This difference is, however, not statistically significant.

In the limb with *chronic* arterial occlusion, the mean collateral resistance at maximal vasodilatation was 0.99 after five weeks of training as compared with 1.37 in the untrained animals. This difference is statistically highly significant ($0.01 > p > 0.001$) (Table I and Fig. 3).

The flow resistance at maximal dilatation in the local vascular bed, placed beyond the collaterals (PRU_{local}, Table I and Fig. 3), was the same immediately



Fig 2 Fig 3

Fig 2. Blood flow resistance of maximally dilated collateral essels five weeks after the arterial occlusion in trained nd trained cats Individual values mean (————) and ±2 S D ( ) are given

Fig 3. Blood flow resistance t maximal dilatation immediately after (A) and fi eeks after (B) the rterial oc lusion

t occlusion and five weeks later There was no difference between trained and rained animals

Muscl at ophy and hypertrophy

The mean muscle weight of th calf in the limb with chronic arterial occlusion was 5 0 per cent less than on the contralateral side. The difference was the same for trained and ntrained animals, and is statistically highly significant ($0.001 > p$) This atrophy due to arterial occlusion was still present when a sham operation was performed in the limb in which the arterial connections were left intact.

The muscle weight of the calf in the trained group when related to body weight. was about 3 per cent highe than in the ntrained. This difference is not significant.

Discussion

Methodological considerat n

In the present experiments th meas ment of collateral and local flow resistances of the hindlimbs was performed during maximal vasodilatation, both to avoid the hazards of changes in vascular smooth muscle tone and to ensure that a truly struc

tural change of the vessels had taken place. For this purpose acetylcholine was infused intra-arterially in increasing amounts until the collateral and local resistance could not be further decreased. These lowest resistance values were often reached when 2 to 5 µg acetylcholine per min was administered to the hindquarters. The fact that the dose-response relationship for acetylcholine could be shown to level off is a good indication that a truly maximal vasodilatation had been achieved. This is also supported by the fact that the local flow resistance could not be further decreased by a period of very intense exercise.

The blood flow was measured as the effluent from the cannulated femoral vein just proximal to the adductor channel, leaving venous collaterals in the surrounding tissues intact. This was necessary since a mass ligature of the tissues would have interfered with the arterial collaterals as well. It has, however, been shown that the major fraction of the blood flow passing through the calf muscles and the distal muscular portions of the thigh, adjacent to the knee, is measured in such a preparation (Thulesius 1962).

The intravascular distending pressure must also be considered in the present comparison of the change in the collateral and local resistances, since even maximally dilated vessels are to some extent distensible, at least in the lower pressure range (Folkow and Löfving 1956). However, the distending pressure for the *collateral* vascular compartment did not differ more than a few mm Hg between the trained and untrained groups (systemic and local pressures for the trained group was 98.4 and 57.2 mm Hg, and for the untrained group 103.7 and 28 mm Hg). These circumstances ensure that the changes in collateral flow resistance, to be discussed below, could not simply be ascribed to differences in the regional distending pressure. Further, the distending pressure for the local vascular compartment was about 15 mm Hg higher 5 weeks after arterial occlusion compared to the situation immediately after the occlusion. Despite this, the local vascular resistance was largely the same in the acute and chronic situation, showing that any outgrowth of the local vascular bed could hardly have taken place. Therefore, from the methodological point of view, it appears as if the present experiments correctly reflect whether, and to what an extent, changes had taken place in the collateral and local vascular compartments.

The collateral resistance

It is obvious that the spontaneous growth of the collateral vessels can be quite extensive. However, physical exercise is evidently a potent additional stimulus for the development of collateral vessels, since the trained animals showed about 50 per cent less collateral resistance as compared to the untrained in the present experiments. It should be stressed that the enforced exercise might after all imply a *fairly moderate* increase of the average work load to which the limb muscles were exposed by the spontaneous activity of the animals even when they were kept in small cages.

The present findings are in good agreement with Eckstein's observations on the

coronary vessels. His method is, however not free from objections (Linder 1966). The reason why an effect of training could be revealed by the method used in Eckstein's experiments is probably due to the fact that retrograde flow from the cannulated distal end of an artery essentially reflects the level of the local arterial pressure. This pressure, other factors being equal no doubt becomes raised when collateral vessels are widened.

The local resistance

In marked contrast to the considerable "spontaneous" development of the collateral compartment, there was no sign of any "spontaneous" outgrowth of the local vascular compartment placed beyond the collaterals. Further the resistance at maximal vasodilatation of this local vascular compartment was the same whether the animals had been trained or not. This latter finding is in agreement with the observations by Grimby Häggendal and Saltin (1967) who found no difference between maximal blood flow per 100 g of muscle in a group of well-trained athletes when compared with untrained persons. Morphologic studies of the number of capillaries in hypertrophy of skeletal muscle and myocardium have given divergent results (Petrén, Sjostrand and Sylvén 1936, Hakkila 1955). The capillaries, however contribute only little to total flow resistance and our studies give no information about that section of the muscle vascular bed.

It thus can be concluded from the present experiments that the outgrowth of the vascular bed after obstruction of a major artery is essentially confined to the collaterals overbridging the occlusion, and that exercise aids in the development of these collateral connections. On the other hand, after training patients with intermittent claudication Larsen and Lassen (1966) could not demonstrate any significant increase in maximal blood flow as measured by the Xe^{133} method though the patients walking tolerance increased. However this type of flow measurement will for several reasons hardly be able to reveal small but nevertheless important improvements of the muscle blood supply. Among other things, the calf muscles are constituted by both "red" and "white" muscle fibres, intermingled to a varying extent in different muscles. It seems as if the blood supply to these two types of muscle is considerably different (see e.g. Folkow and Halicka 1968). The flow values deduced from the clearance of intramuscularly injected isotopes may therefore to a considerable extent vary with the local proportion of red and white muscle units. Further a measurement of the blood flow reflects the sum of the collateral and local resistances, which makes it more difficult to reveal changes in the collateral vessels alone. The increased walking tolerance after training might also be due to a higher pain tolerance, changed local distribution of blood within the ischemic muscle or learning of more economic walking pattern.

Changes in muscle weight

The intensity and duration of training used in the present experiments, is comparable to that used in the study by Eckstein (1957). The increase in muscle and

heart weight, respectively is of the same order when related to body weight, i.e. about 3 per cent. In the material of Petrén, Sjöstrand and Sylvén (1936) the heart size per body weight was about 45 per cent higher after training. It should be observed that different species were used in the three investigations and that Petrén, Sjöstrand and Sylvén (1936) used growing animals.

There was a significant muscle atrophy 5 weeks after the arterial occlusion. This atrophy was probably not due to inactivity because it was equal in trained and untrained cats and the trained animals showed no signs of intermittent claudication, nor did a sham operation on the contralateral side affect the results.

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Hemodynamics of the Bone Marrow Circulation

By

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Abstract

MICHELSEN K. *Hemodynamics of the bone marrow circulation* Acta physiol. scand. 1968 73 264—280.

In 44 anesthetized rabbits the femur was perfused in sit through the nutrient artery by blood led through an extracorporal perfusion arrangement from the left carotid artery Blood inflow perfusion pressure and intramedullary enous pressure were recorded. At perfusion pressures of 110 t 150 cm of saline (80 to 110 mm of Hg) blood flow in the different animals ranged from 0.1 to 1.2 ml/min×g wet marrow tissue and intramedullary enous pressure from 10 to 80 cm of saline (7 t 60 mm of Hg) Flow and intramed llary enous pressure both increased with the perfusion pressure whereas flow decreased and intramedullary enous pressure increased when the enous pressure outside the bone increased, showing that in the bone marrow both n arterial and a enous resistance were present. The predominant effect of noradrenaline was t constrict, and of acetylcholine to dilat arterial vessels. N eactive hyperemia could be demonstrated pon occlusion of the arterial inflow The arterial vessels showed only weak ability to autoregulate flow The enous resistance ried greatly from one reparation to another differing from only a small fraction of—t more than half the total ula resistance the bone marrow It stayed constant or increased with increasi g perfu- ressure, thereby causing the pressure-flow relationship curves to diffe from convex the pressure—to convex against the flow axis. The hemodynamic properties demon d were explained as being due to the unusual vascular anatomy and the rigid enclos- of the bone marrow

The pressure relationships in the bone marrow vascular bed have been studied in a previous work (Michelsen 1967) The results indicated that the tissue pressure and the pressures in the vascular channels past the arterioles were about equal within a region of the bone marrow cav ty This pressure aried between 10 and 80 cm of saline and pulsated in synchrony with the arterial blood pressure, showing that there must be import nt differences between the hemodynamics of bone marrow circulation and that of most other tissues. In order to further elucidate bone marrow hemodynamics it was necessary to correlat tissue- and vascular pressures with the blood flow

Many methods for blood flow ontrol nd measurement have been applied to the bone marrow circulation, but for several reasons none of them have proved entirely satisfactory The bone marrow is thus supplied not by one, but by sev ral arteries, and is drained by several eins. Furthermore the intramedullary arteries supply not

only the bone marrow but they send off branches to the osseous tissue as well (Brookes and Harrison 1957 Brookes 1958) Finally the bone marrow tissue is exceptionally fragile.

Thermo-electric methods, which have been used by several workers for evaluation f blood flow in the bone marrow (Braunsteiner and Grabner 1958, McPherson Scales and Gordon 1961 Shaw 1963 1964) have yielded valuable information. However these methods may be criticised. They gi e qualitative information only and their use necessitates injury to bone marrow tissue. Since blood flow can occur through damaged bone marrow (Tocantins and O Neill 1941 Sternbach *et al.* 1957 Sime 1956 Dickerson and Duthie 1963) the measured blood flow will be influenced to an unknown degree by bnormal flow through the lesions in this ulnerable and rigidly enclosed tissue (Michelsen 1967)

The bone marrow blood flow has been evaluated by measurement of the clearance rate for an isotope injected directly into a region of the bone marrow (Petrakis, Masouredis and Miller 1953 Brown-Grant and Cumming 1962, Najean and Clement 1963) However since bone marrow tissue is injured by the injection, an unknown and variable fraction of the injected isotope may pass directly into the venous system (Brodin 1955)

Breuer Hirsch and Sach eh (1964) applied the Fick principle for flow determination in th bone marrow of the rabbit femur using amino-antipyrin as indicator substance (Huckabee and Walcott 1960) The amino-antipyrin concentrations were measured repeatedly in the arterial blood and in blood from the nutrient ein of the femur the time ersus concentration curves were drawn, and the general Fick equation applied to calculate flow Except for the cannulation of the nutrient ein the bone marrow circulation was not interfered with. This method is probably the best one available for a limited number of determinations of the bone marrow blood flow during stable conditions. However t is not suitable for an analysis of dynamic aspects.

Cumming (1960 1962) and Cumming and Nutt (1962) estimated the blood flow in the rabbit femur by method of venous effluent collection. All branches of the femoral rtery and vein except the nutrient artery and vein of the femur were ligated and the outflow in the femoral ein was measured. A great advantage of this method is that the blood is not interfered with until t lea es the bone marrow An important objection is that the bone marrow is drained by many veins other than the nutrient ein, especially in the metaphyseal regions. Taking this into account, Post and Shoemaker (1964) dissected out all veins draining the fem in the dog in order to measure the total enous outflow However this method requires rather extensive surgical procedures, and the measured enous blood flow will includ the outflow also from osseous tissue.

Control of blood flow through a region can also be achieved by perfusion rrangements. Perfusion with blood through the n trient artery of bones has previously been performed in the dog by Drinker Drinker and Lund (1922) These investigators devoted their interest mainly to hematological problems. In 1962 Held and Thron

performed a pressure flow study by perfusion through the nutrient artery of the tibia of the dog.

In the present work blood flow through the bone marrow vascular bed of the rabbit femur has been controlled with a perfusion arrangement. The blood was taken *from the left carotid artery and pumped through an extra-corporal circuit* where flow was measured, and then into the bone marrow through the nutrient artery. Such a procedure may change the properties of the blood as regards its influence upon vascular smooth muscles (Folkow 1952). It may also be argued that the bone marrow is normally supplied by several arteries and that the nutrient artery send off branches also to the osseous tissue. In the rabbit femur however arteries other than the nutrient artery are probably of minor importance for the supply of the bone marrow (de Marneffe 1951) and the nutrient artery is mainly supplying bone marrow tissue (Cumming 1962). In spite of the objections that can be made against the in situ perfusion method used in these experiments, it was judged as the best available method for an analysis of the basic hemodynamic properties of the bone marrow circulation.

Methods

The experiments were carried out on the left femur of the rabbit. 44 animals of both sexes, of varying age and weighing between 3.0 and 5.0 kg, were used. They were anesthetized by i. v. injection of pentobarbitone (Nembutal® Abbott) in an initial dose of 30 to 50 mg/kg with maintenance doses of 20 to 30 mg/kg×hr. The animals were tracheotomized and ventilated by positive pressure ventilation. They w re lying on the right side with the left hindlimb loosely fastened in semiflexion the hip and knee joints.

The systemic arterial pressure was measured through an indwelling catheter in the right femoral artery. This and other pressures were recorded with pressure transducers (Statham P 23 De) on multichannel recorder (Sanborn Company Mass., U.S.A.)

lood coagulation was prevented by i travenous injection of heparin (500 i.u./kg b.wt.) rombocyte aggregation was avoided by initial removement of thrombocytes glass fiber lem 1960 as follows. Blood from the lef carotid artery was pumped through fiber pring of 10 g of glass beads, 0.5 mm in diameter whereafter the blood was returned to e animal in the right jugular ein. The flow through the filter was 15 ml/min. The proced re was carried out for about 30 min and prior to start of the bone marrow perfusion.

Perfusion arrangement d method for measurement f flow. Fig. 1A is diagram of the perfusion arrangement. Arterial blood from catheter in the left carotid artery passed through a peristaltic pump (Model 500—1200 M Harvard apparatus Co. Dover Mass U.S.A.). A ariable f action of he blood went through stiff-walled polyethylene tubing 30 cm long and with an inner diameter of 0.6 mm. The pressure difference (P — P = ΔP) between the two ends of this bing was measured by differential transducer (Statham P 23 H). As the resistance to flow the tubing stayed constant, the flow through the tubing was bout proportional the pressure differenc measured. From the end of the tubing the blood entered into catheter in the nutrient artery of the femur. The pressure (Ps) t the distal end of the stiff-walled polyethylene tubing was recorded and the perfusion pressure in the nutrient artery calculated by subtracting from this P the pressure fall through the catheter in the nutrient artery the arious flow rates. Determination of this pressure fall and calibration of the flow recording were carried ou the end of each experiment.

The remaining and aryin fraction of the blood from the pump did not pass the flow recording device, bu went hrough Starling resistance (Renkin 1962) to the right jugular ein of the rabbit. The Starling sistance consisted of wide flaccid rubber tubing within plastic cylinder. The perfusion pressure and thereby also the fraction of the pumped blood which went into the nutrient arter ld be altered by changing the pressure in the Starling resistance.

(Since flow is measured as pressure fall (P — Ps) through a constant resistance decrease in bone marrow flow at constant pressure in the Starling resistance will necessarily increase the perfusion pressure (Ps).

A

Right jugular vein

Left carotid artery

To the nutrient artery

Tubing with constant resistance to flow

Water coat

Water 38°C

Peristaltic pump

Differential transducer

Perfusion pressure

Flow

Storing resistance

B

Catheter in the nutrient artery

Catheter in the nutrient vein

Nutrient foramen

Fig 1 A. Diagram of arrangement for perfusing rabbit femurs in situ through the nutrient artery
B. The nutrient artery and vein of the rabbit femur after insertion of tubings into these vessels.

The flow recordings presented give the impression that the blood flow was pulsatory. This is due to the effect of the peristaltic pump on the P_1 pressure. In the nutrient artery the pulsations were insignificant.)

The polyethylene tubing for flow measurement was enclosed in a wider tubing which was perfused with water of 38° C, so that the blood had this temperature when it entered the nutrient artery.

Operative techniques. An incision was made through the skin on the antero-medial side of the left thigh from the inguinal ligament to the knee region. All medially directed ramifications of the femoral vessels were ligated and cut, and the common fascia around the thigh muscles was split along the medial border of the femoral vessels. By means of a wound holder the adductor muscles of the thigh were pressed dorsomedially and the quadriceps muscle with the femoral vessels laterally. The nutrient artery and vein were thereby exposed (Fig. 1B). These vessels came off from the root of the lateral circumflex vessels on the anterior side of the thigh, about 0.5 cm distal to the inguinal ligament. They ran distally and backwards for about 2 cm in a sheet of fat before they entered the nutrient canal on the medial side of the femoral shaft in front of the lesser trochanter. Both vessels had a varying number of ramifications. The nutrient artery had a diameter between 0.4 and 0.7 mm and the nutrient vein a diameter between 0.5 and 0.9 mm. Under a dissecting microscope all their ramifications were tied off and a 10 mm long polyethylene tubing with an outer diameter of 0.6 mm was inserted into the nutrient artery and another into the nutrient vein. The catheter tips were positioned 0.5 to 1.0 mm from the nutrient foramen. The catheter in the nutrient artery was connected to the perfusion system and the catheter in the nutrient vein to a pressure transducer for measurement of the intramedullary venous pressure. A thin nerve accompanying the nutrient vessels was cut.

In 10 expts. the intramedullary venous pressure was measured as bone marrow pressure

method for measurement of bone marrow pressure has been described and its validity discussed in a previous publication (Michelsen 1967).

The cannulation of the nutrient artery and vein required about 20 min. During this period no blood went through the nutrient artery.

The perfusion of the bone marrow was started less than one hr after anesthetizing the rabbit and was carried out for less than 3 hrs.

Noradrenaline and acetylcholine infusion. Noradrenaline (Nor-exadrin® Astra) and acetylcholine chloride (Acetylcholine® Roche) could be added to the nutrient artery blood flow through a side-branch in the perfusion arrangement past the side-branch for measurement of the perfusion pressure (Pa) and 1 cm proximal to the tubing in the nutrient artery. Both substances were diluted in saline to a concentration of 1 mg/ml. The drugs were infused for various lengths of time by an infusion pump (Type 1830 B. Braun, Apparatebau, Melsungen, W-Germany) at rates between 0.01 and 0.05 ml/min. Noradrenaline could also be injected through a catheter in an ear vein.

Macroscopical and histological examination. At the end of each experiment the femoral bone was cleaned of soft tissue and weighed. The bone was carefully crushed. The marrow tissue taken out weighed, examined macroscopically and in 10 cases also histologically (hematoxylin and eosin staining).

Results

The weight of the individual femurs varied from 10.2 to 15.0 g. The amount of bone marrow tissue per femur varied from 1.5 to 2.5 g wet weight. The femoral bone marrow cavity was smooth-walled without trabecular bone except in the proximal end and in the femoral chondyles. The marrow tissue was easily separated from the osseous tissue.

Macroscopical and histological examination of the bone marrow revealed a varying mixture of red and yellow marrow tissue with one or the other as the dominant component. Yellow marrow was usually found centroaxially in the bone marrow cavity. Occasionally regions with pure yellow marrow were present in between regions with a mixture of red and yellow marrow tissue especially in the distal epiphysis. Histological examination after perfusion showed no difference between the perfused left femoral bone marrow and the right femoral bone marrow which had not been experimentally interfered with.

The blood flow through the nutrient artery at perfusion pressures near the systemic blood pressure of the respective rabbits differed considerably from one experiment to another. In 44 experiments where the perfusion pressure was kept between 110 and 150 cm of saline (81 and 110 mm of Hg) did the flow values range from 0.1 to 1.2 ml/min×g wet marrow tissue (Fig. 2). The blood flow was small in animals where the femoral bone marrow tissue was dominantly yellow as judged from the macroscopical and histological examination. It was great in animals with a dominantly red femoral bone marrow.

The intramedullary venous pressure at a nutrient artery perfusion pressure between 110 and 150 cm of saline did also differ from one experiment to another. Values between 10 and 80 cm of saline were recorded. Such differences were present independent of whether the intramedullary venous pressure was measured through the nutrient vein or as bone marrow pressure. When the intramedullary venous pressure was low the blood flow was often, but not always, great.

Changes in the bone marrow flow were usually accompanied by changes in the

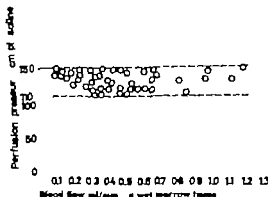


Fig. 2. Blood flow through the nutrient artery of in situ perfused rabbit femurs at perfusion pressures in the nutrient artery between 110 and 150 cm of saline. Values from 44 experiments given in ml/min×g wet marrow tissue.

intramedullary venous pressure. The two parameters could change in the same as well as in opposite directions. An increase in the perfusion pressure caused an increase both in flow and in intramedullary venous pressure (Fig. 3A, B), whereas application of a tourniquet around the thigh led to an increase in the intramedullary venous pressure and to a decrease in flow (Fig. 3C). Spontaneous and simultaneous changes in the two parameters, sometimes in the same and sometimes in opposite directions, were also seen in the course of an experiment. These spontaneous changes took place gradually in the course of minutes.

The effects on the bone marrow hemodynamics of a vasoconstrictor and of a vasodilator agent were tested by addition of noradrenaline and acetylcholine to the blood inflow through the nutrient artery. Addition of noradrenaline (1.6 to 4.8 μg/10 sec) to the nutrient artery inflow caused both flow and intramedullary venous

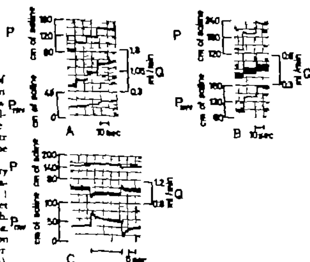


Fig. 3 A and B. The effect of stepwise changes in perfusion pressure (P) on bone marrow blood flow (Q) and intramedullary venous pressure (P_{mv}). Recordings from two rabbit femur preparations perfused through the nutrient artery.
C. Changes in nutrient artery blood flow (Q) and in intramedullary venous pressure (P_{mv}) on application of a tourniquet around the middle of the thigh. Tourniquet present during ←. The changes in the perfusion pressure (P) are due to the perfusion arrangement (see methods).

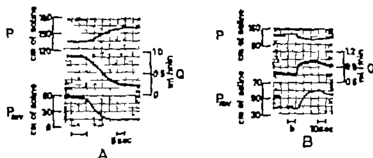


Fig. 4 The effect of noradrenaline and acetylcholine on the blood flow (Q) and the intramedullary venous pressure (P_{mv}) in the rabbit femoral bone marrow perfused through the nutrient artery. The change in the perfusion pressure (P) is due to the perfusion arrangement (see methods).
A During infusion of noradrenaline at rate of 10 μg/min.
B. During *b* infusion of acetylcholine at rate of 10 μg/min.

pressure to decrease markedly (Fig 4A) The effect of infused noradrenaline lasted for from 3 to 10 min. The magnitude and duration increased with the infused dose. Large doses caused the blood flow to drop to zero and lowered the intramedullary venous pressure to levels between 10 and 20 cm of saline.

Acetylcholine (1 6 to 4 8 μg 10 sec) caused an increase in flow and intramedullary venous pressure (Fig 4B) The effects of acetylcholine were pronounced when the intramedullary venous pressure was low before the infusion. In such cases the blood flow could be increased two- to threefold and the intramedullary venous pressure elevated by 20 to 60 cm of saline When the intramedullary venous pressure was high before the acetylcholine infusion, the effect on the blood flow was small whereas the effect on the intramedullary venous pressure was great.

In order to determine whether the bone marrow vascular bed shows a reactive hyperemia following a temporary occlusion of the arterial blood supply the blood inflow through the nutrient artery was occluded for 30 to 120 sec and then reestablished. Fig. 5A shows one typical experiment of this type. During the first sec of reestablished blood supply subsequent to the arterial occlusion, the blood flow increased sharply whereas the intramedullary venous pressure remained low Thereafter the blood flow decreased and the intramedullary venous pressure increased both transiently attaining values below their preocclusion values, to which they slowly returned in the course of 1 to 5 min.

In 7 expts. a small retrograd blood flow was observed from the nutrient artery catheter when this was opened to the atmosphere. This illustrates that in these preparations arterial anastomoses were present between the ramifications of the nutrient artery and arteries that nt the bone marrow in the metaphyses (Brookes and Harrison 1957) In these cases the nutrient artery pressure at zero flow was between 25 and 50 cm of saline

The influence of such arterial anastomoses on the bone marrow hemodynamics is illustrated in Fig 5B which is a recording of the effects of noradrenaline injection

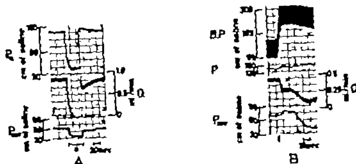


Fig. 3 A. Changes in perfusion pressure (P) bone marrow blood flow (Q) and in intramedullary enous pressure (P_{iv}) during od after a temporary stop (40 sec) In the blood flow through the nutrient artery Rabbit femur preparation perfused through the nutrient artery
B The effect of generally administered noradrenaline in perfused rabbit femur preparation ith anastomoses present between the ramifications of the utrient artery and the metaphyseal arteries (see text) Recordings of systemic blood pressure (B.P) nutrient artery perfusion pressure (P) and blood flow (Q) and intramedullary enous pressure (P_{iv}) At (x) was 50 µg of noradrenaline injected through an ear vein.

in an experiment in which a retrograde blood flow was present through the nutrient artery

At (x) 50 µg of noradrenaline was injected through an ear vein. The systemic blood pressure increased, and at the same time the blood inflow through the nutrient artery decreased whereas the intramedullary enous pressure increased. Some sec later both the blood flow through the nutrient artery and the intramedullary venous pressure decreased greatly As will be discussed later these changes can be interpreted as due to vascular effects of noradrenaline and to the presence of anastomoses between ramifications of the nutrient artery and arteries that enter the bone marrow n the metaphyses

Though a change in the perfusion pressure caused a change in the same direction of both flow and intramedullary venous pressure the magnitude of the ffect on the intramedullary enous pressure varied considerably In some cases the intramedullary enous pressure changed only a little as compared to the perfusion pressure change (Fig 3A) In such cases the bone marrow blood flow was usually high. In other cases the intramedullary venous pressure was more markedly influenced by the perfusion pressure (Sometimes the two pressures even changed about equally much) The blood flow was then usually small and changed only a little upon changes in the perfusion pressure (Fig 3B)

These differences between preparations in the behaviour of the intramedullary enous pressure expressed themselves n the relationship between steady state alues of perfusion pressure intramedullary enous pressure and blood flow Fig 6A B C and D show such relationships from four different experiments. The flow Q) has been plotted ersus the perfusion pressure (P) versus the intramedullary venous pressure (P_{iv}) and ersus the difference between these two pressures (P

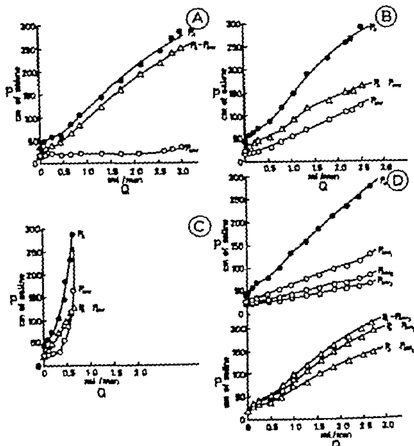


Fig 6 A, B, C and D Pressure versus flow relationships from 4 different rabbit femur preparations perfused through the nutrient artery Flow (Q) has been plotted versus perfusion pressure in the nutrient artery (P) versus intramedullary venous pressure (P_m) and versus the pressure difference between perfusion pressure and intramedullary venous pressure (P_a—P_m). The perfusion pressures have been corrigated for the pressure fall in the nutrient artery cannula. In Figs. A, B and C the intramedullary venous pressure was measured in the nutrient vein, in Fig. D as bone marrow pressure in the mid-diaphysis (P_{mv1}), in the distal metaphysis (P_{mv}) and in the proximal metaphysis (P_{mv3})

Fig 6A, B and C the intramedullary venous pressure has been measured through the nutrient vein, in Fig 6D as bone marrow pressure in the diaphysis (P_{mv1}) in the distal metaphysis (P_{mv2}) and in the proximal metaphysis (P_{mv3})

At zero flow the pressure in the nutrient artery varied from 20 to 40 cm of saline. It was from 10 to 30 cm higher than the intramedullary venous pressure

In Fig 6A the perfusion pressure (P) versus flow curve is slightly convex against the flow axis in the pressure range between 60 and 140 cm of saline. Above 140 cm it becomes convex against the pressure axis. The intramedullary venous pressure (P_{mv}) increased only insignificantly with increasing flow and perfusion pressure in this experiment. The P—P_{mv} versus flow curve has the same shape as the perfusion pressure versus flow curve

In the experiment of Fig 6B the perfusion pressure—flow relationship gave a curve slightly convex against the flow axis below a perfusion pressure of 150 cm of saline, above this pressure the curve was convex against the pressure axis. The intra medullary venous pressure versus flow curve is about linear whereas the P —P_{mv} versus flow curve is slightly convex against the flow axis in the lower pressure range and is moderately convex against the pressure axis in the higher pressure range

In Fig. 6C the perfusion pressure—flow curve is about linear at a perfusion pressure below 80 cm of saline. Beyond this perfusion pressure the curve is strongly convex against the flow axis. The intramedullary venous pressure was low when the perfusion pressure was below 80 cm of saline but above this perfusion pressure the venous pressure increased greatly The P —P_{m} versus flow curve is about linear in the whole pressure range tested. It should be noted that the P —P_{mv} difference increased only insignificantly as compared to the perfusion pressure in the higher per fusion pressure range. The maximal P —P_{mv} difference was 130 cm of saline in this experiment.

Fig. 6D shows that though the intramedullary venous pressure is not the same in the different regions of the bone marrow cavity the shape of the intramedullary venous pressure (P_{mv}) versus flow curves and of the P —P_{mv} versus flow curves is the same for all regions.

In the present series of 44 experiments, clearcut pressure versus flow relationships of the type illustrated in Fig. 6A were present in 10 preparations and of the type illustrated in Fig. 6C in 8 preparations. The rest of the preparations showed relationships which were similar to the one illustrated in Fig 6B, though often with some resemblance to either the type shown in Fig 6A or to that shown in Fig 6C.

Discussion

The intention with these experiments has been to reveal the basic hemodynamic properties of the bone marrow circulatory system with its rigid enclosement and peculiar vascular anatomy Since the experimental procedures were extensive and included the use of a perfusion pump and since the bone marrow is not normally exposed to such extreme pressure and flow variations as those used here, it is not stated that the properties found in these experiments necessarily manifest themselves also in the intact animals.

As illustrated in Fig th nutrient artery blood flow per gram wet marrow tissue at perfusion pressures close to the arterial blood pressures of the experimental animals differed greatly from one animal to another indicating that the vascular resistance per g wet marrow tissue differed considerably Similar differences in bone marrow blood flow were also found by Cumming (1962) with his method of venous effluent collection. He suggested that they were due to variations in hematopoietic activity as the blood flow was found to be great in young animals with a high cell larity of the bone marrow In the present experiments, flow was also found to be high when red marrow tissue dominated. Breuer and Hirsch (1964) using the

amino-antipyrin method for flow determination, reported similar relationships between the bone marrow blood flow and hematopoietic activity and Linke *et al.* (1965) found a high blood flow in rabbits which had been made anemic.

Not only blood flow but also the intramedullary venous pressure differed considerably from one animal to another at perfusion pressures which were close to the arterial blood pressure of the experimental animals. Furthermore it was found that both the blood flow and the intramedullary venous pressure increased though to a greatly varying extent with the perfusion pressure (Fig 3A and B) whereas application of a tourniquet which imposed impediment to the venous drainage (Fig 3C) *caused a decrease in flow and an increase in the intramedullary venous pressure.*

These findings imply that in the bone marrow circulatory system there is often a considerable venous as well as an arterial resistance. The blood flow through a region of the bone marrow should then be determined by the equations

I) $Q = (P - P_m)/R_a$ II) $Q = (P_{mv} - P)/R_v$

(P = arterial blood pressure here perfusion pressure

P = venous pressure in great collecting veins outside the bone

P_m = intramedullary venous pressure and tissue pressure, in a region of the bone marrow

Q = blood flow through this bone marrow region,

R_a = *arterial resistance to blood flow into the region,*

R_v = venous resistance to blood *flow* between the region and the great *collecting* veins.)

If the *intramedullary venous pressure had been the same throughout the bone marrow cavity the total arterial and the total venous resistance could have been* termined by application of equations I and II to the bone marrow cavity *as a* Since however the intramedullary venous pressure often differed in the rious regions along the long axis of the bone marrow cavity but was usually known only in one region in these experiments, the total arterial and venous resistance can not be determined quantitatively from the data presented. However the relationship between flow and intramedullary venous pressure and between flow and *the pressure difference $P - P_m$ were similar for the different bone marrow regions* (Fig 6D) *By application of equations I and II where Q now is the total blood flow* and P_{mv} a regional venous pressure, it is therefore possible to analyse the arterial and the venous resistance qualitatively

The arterial sistan In the first part of their intramedullary course the arteries of the bone marrow vascular bed have an endothelial, a muscular and an adventitial layer However soon after the branching, the arteries lose their muscular coat abruptly and in the further course the walls of these vessels consist only of an endothelial and an adventitial layer (*Hashimoto* 1936, *Yoffey* 1965) From these thin-walled arteries by some authors called arterial capillaries, *the sinusoids* take off at right angels.

One must presume that active constriction and dilatation of arterial vessels can

take place only in the thick walled arteries, which possess smooth muscles. Since there are no precapillary sphincters in the bone marrow vascular bed, it appears that the vasomotor control of this circulation must differ from that of other vascular areas. However marked effects on the bone marrow vascular resistance could be obtained by noradrenaline and acetylcholine (Fig 4A and B) The drop in blood flow and the increase in th P —P_{av} difference caused by noradrenaline show that this substance greatly increased the arterial resistance in the bone marrow and with large doses it was possible to stop the bone marrow blood flow completely

Acetylcholine causing an increase in blood flow and a decrease in the P —P_{av} difference, reduced the arterial resistance considerably (Fig. 4B) The maximal decrease in the arterial resistance which could be produced by acetylcholine varied considerably from one experiment to another indicating that the vascular tone in the arterial vessels of various femoral bone marrows differed. Such differences may well be present also in the intact animal. However it can not be excluded that the passage of blood through a pump device (Folkow 1952) had caused different degrees of reduction in the vascular tone in the present preparations.

When the arterial blood supply was restituted after a temporary occlusion, the blood flow increased instantaneously However it stayed below the preocclusion level for several minutes and in this period the P —P_{av} difference was somewhat greater than before the occlusion. It can therefore be concluded that the arterial resistance was increased and not decreased in the postocclusion period. It may be argued that this lack of reactive hyperemia could be due to reduction or elimination of vascular tone in these preparations. The reduction in arterial resistance which was caused by acetylcholine shows, however that a vascular tone was present. A reactive hyperemia should therefore have manifested itself if present. It may also be questioned whether the nutrient artery vascular bed, during the occlusion of the nutrient artery inflow was not supplied through arterial anastomoses from arteries which entered the bone marrow in the metaphyseal regions. However as will be discussed below it seems that the blood supply through such arterial anastomoses was usually insignificant

The lack of a reactive hyperemia in these experiments are in accordance with the finding in a previous work (Michelsen 1967) were it was shown that after a temporary clamping of the arteries to rabbit hind limb the tibial bone marrow pressure did not increase beyond the preclamping level. Such an increase would have been expected if a reactive hyperemia had developed. Whether this lack of a reactive hyperemia in the bone marrow vascular bed is due to the unusual vascular anatomy in this tissue or to local biochemical or biophysical changes not developing as in other vascular beds, can not be determined from these experiments.

At zero flow the pressure in the nutrient artery was 10 to 30 cm of saline higher than the intramedullary venous pressure. In the majority of the experiments, there was no retrograde blood flow through the nutrient artery and in these cases this pressure difference indicated that the bone marrow arterioles were closed Arterial anastomoses between branches of the nutrient artery and arteries which the

bone marrow in the metaphyses (Brookes and Harrison 1957) were apparently functionally important in 7 experiments, in which there were a retrograde blood flow through the tubing in the nutrient artery when this was opened to atmosphere. These arterial anastomoses will influence the blood flow through the nutrient artery during perfusion. At nutrient artery perfusion pressures below the systemic blood pressure the blood flow through the nutrient artery will be less than if anastomoses were not present, as the anastomosing arteries will supply some blood to the nutrient artery vascular bed. At perfusion pressures above the systemic blood pressure the flow through the nutrient artery will be greater than if anastomoses were not present, since the nutrient artery will then supply blood to the vascular bed of the anastomosing arteries. In addition, the blood supply through metaphyseal arteries will influence the intramedullary venous pressure and also thereby the blood flow through the nutrient artery. These effects are illustrated in Fig. 5B. The rise in the systemic blood pressure (B.P.) caused by injection of noradrenaline (at x) increased the blood pressure in the metaphyseal bone marrow arteries beyond the perfusion pressure (P) in the nutrient artery causing the blood flow (Q) through the nutrient artery to drop. At the same time the intramedullary venous pressure (P_m) rose, indicating that the total blood flow to the bone marrow was increased. Some seconds later both the bone marrow blood flow and intramedullary venous pressure decreased, because noradrenaline then reached the bone marrow vascular bed and caused constriction of its arterial vessels.

When a metaphyseal blood supply is present in addition to that through the nutrient artery, quantitative considerations should be performed with care. The qualitative hemodynamic considerations will, however, not be invalidated.

In the pressure flow relationship curves presented in Fig. 6A, B, C and D the [illegible]ial resistance can be evaluated from the curves representing the relationship between flow and the difference between perfusion pressure and intramedullary venous pressure ($P - P_m$). Since intramedullary tissue pressure and venous pressure are about equal (Michelsen 1967), the $P - P_m$ difference will also represent the difference between perfusion pressure and intramedullary tissue pressure, and it is therefore possible to evaluate also the distending forces in the bone marrow arterial vessels from the $P - P_m$ versus flow relationship.

A comparison of the $P - P_m$ curves in Fig. 6A, B and C tells that the arterial resistance behaved similarly as a function of the distending forces in the arterial vessels. The convexity of the $P - P_m$ curves against the flow axis in the pressure range between 60 and 140 cm (Fig. 6A and B) shows that the arterial resistance increased somewhat with increasing transmural pressures. Above 140 cm of saline the curves are convex against the pressure axis and the arterial resistance thus decreased with increasing transmural pressures in the higher pressure range. The convexity of the curves against the flow axis between 60 and 140 cm of saline shows that the arterial vessels exhibited a weak autoregulation of blood flow in these experiments.

It is possible that a weak autoregulation is a peculiarity of bone marrow arterial

vessels, and it should be noted that Held and Thron (1962) found no signs of autoregulation in a pressure flow analysis of the femoral bone marrow of the dog. However, it can not be excluded that a normally marked autoregulation has been masked by a decreased vascular smooth reactivity in these preparations. It might also be questioned whether a pronounced autoregulation could have been masked by blood flow through arterial anastomoses between the ramifications of the nutrient artery and metaphyseal arteries. However, as discussed above, such arterial anastomoses seemed to be functionally insignificant in most preparations.

The venous resistance. The intramedullary venous system consists of a central venous channel which runs from one end of the bone marrow cavity to the other (Brookes and Harrison 1957, de Marneffe 1951), by means of radiating venules it drains the sinusoids and capillaries. A nutrient vein and several veins of great calibre especially in the metaphyseal regions, connect the central venous channel to the outside venous system. The walls of the intramedullary veins consist of endothelial cells of the same type as those found in the sinusoids; vascular smooth muscles are not present.

Since the dimensions of the transcortical part of the veins draining the bone marrow are great (de Marneffe 1951) and no sphincter-like structures have been described in the draining veins, the venous resistance to bone marrow blood flow must be situated within the bone marrow and must depend on the transsectional dimensions of the intramedullary veins. When the venous resistance is small, the transsectional dimensions of the intramedullary venous system must be great, and when the venous resistance is great the transsectional dimensions of the intramedullary venous system must be small either throughout the bone marrow or in some regions. Some of the individual differences observed in the venous resistance seem to be related to different degrees of hematopoietic activity, since the venous resistance has been found to be constantly low in anemic rabbits with a high cellularity of the bone marrow (Michelsen in preparation). However, since the volume of the bone marrow cavity is constant, any increase in the marrow volume outside the intramedullary venous system will lead to a compression of the veins and to an increase in the venous resistance. One type of possible volume expansion outside the venous system is a dilatation of the sinusoids. That the sinusoids can vary in volume in spite of their lack of smooth muscles in the walls, has been shown by Brånemark (1959) in an in vivo microscopical study of the bone marrow of the rabbit fibula. Also a tissue oedema as well as a hemorrhage into the bone marrow tissue could cause compression of intramedullary veins and thereby an increase in the venous resistance. The development of oedema or hemorrhage in some of the experiments could perhaps explain some of the differences found in venous resistance. Although it has not been possible to exclude the development of such conditions in all experiments, this seems to be an unlikely explanation. Thus the preparatory methods were standardized, and histological examination revealed no difference between the bone marrow of the perfused left femur and that of the right femur which had its normal blood supply intact. The finding of a greatly varying venous resistance is

sistent with the observation that the bone marrow pressure differed greatly in animals that had not been experimentally interfered with apart from the anaesthesia and the insertion of a needle into the bone marrow (Cuthbertson, Gilfillan and Bachman 1964 Stein, Morgan and Reynolds 1957)

From an analysis of recordings as those illustrated in Fig 4A and B, it can be concluded that neither noradrenaline nor acetylcholine did significantly change the venous resistance. This is in accordance with the fact that vascular smooth muscles lack in the venous vessels. It also indicates that these drugs do not induce significant changes in the volume of the bone marrow sinusoids, as such changes would alter the volume of the intramedullary venous system and thereby the venous resistance.

During the first sec following restitution of the nutrient artery blood flow after a temporary occlusion the intramedullary venous pressure increased rapidly In the same period of time the blood flow fell (Fig 5A) This shows that immediately after restitution of flow the venous resistance was decreased however it was rapidly reestablished.

In Fig. 6A, B and C the venous resistance can be analysed from the curves representing the relationship between intramedullary venous pressure (P_m) and flow (Q) A comparison between the different P_{mv} curves shows that the venous resistance behaved differently in the various experiments. In the experiment of Fig 6A the venous resistance was very low In the experiment of Fig 6B it stayed about constant whereas in the experiment of Fig. 6C it increased greatly with flow and perfusion pressure.

The differences in the intramedullary venous pressure (Fig 6D) in the diaphysis (P) in the distal metaphysis ($P_{m\,2}$) and in the proximal metaphysis (P_{mv}) ly show that resistances were present between the different regions of the intramedullary venous system. Furthermore, when the intramedullary venous pressure as high a considerable pressure fall must have been present between the intramedullary and the extraosseous part of the draining veins. Since the dimensions of the transcortical part of the draining veins are great, this pressure fall must have been situated close to the entrance of the draining veins into the transcortical channels, requiring a partial collapse of the intramedullary veins at the entrance into the transcortical channels. If the volume of the bone marrow cavity could vary this "collapse resistance" would always passively adjust itself to the flow and the pressure fall. The intramedullary venous pressure would then be influenced neither by a change in flow nor by an increase in the venous pressure outside the bone as long as the latter pressure was below the intramedullary venous pressure. This would mean that a vascular waterfall phenomenon (Permutt, Bromberger Barnea and Bane 1962) were present. However as illustrated in Fig. 6B and C the intramedullary venous pressure increased with flow and in previous work (Michelsen 1967) it was found that an increase in the venous pressure outside the rabbit tibia caused an increase in the tibial bone marrow pressure even when the former was considerably below the latter The explanation is probably that the constant volume of the bone marrow

cavity prevents a dilatation of the partially collapsed veins and thus hinders a decrease in th collapse resistance In some cases the collapse resistance did apparently remain about constant (Fig. 6B) whereas in other cases it increased greatly with increasing perfusion pressure. The intramedullary venous pressure then increased towards the perfusion pressure and the system went towards complete occlusion.

Provided that the arterial and the venous resistance are coupled in series, the total resistance to bone marrow blood flow is determined by the sum of these two resistances. At perfusion pressures close to the normal arterial blood pressures of the experimental animals, the greater part of the total resistance was usually situated in the arterial vessels. It should be noted though that in some experiments, especially t high perfusions pressures, it was the bone marrow venous system that dominated the total vascular resistance to bone marrow blood flow

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Position and Velocity Sensitivity of Muscle Spindles in the Cat. I. Primary and Secondary Endings Deprived of Fusimotor Activation

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Abstract

LENNERSTRAND, G. *Position and velocity sensitivity of muscle spindles in the cat. I. Primary and secondary endings deprived of fusimotor activation.* Acta physiol. scand. 1968. 73. 281—299.

Responses of primary and secondary muscle spindle endings of the ankle extensor and flexor muscles in the cat were studied in the absence of fusimotor activation by applying length changes of triangular form over a large range of velocities. Both position and velocity responses were determined. No differences were obtained in these responses between endings in extensor and flexor muscles or between endings in fast and slow muscles. It was found necessary to subdivide the velocity responses into two different components based on the great difference in *time course of their transients. Mathematical expressions for the two components are given in* an Appendix. They were denoted the 'quick' and the 'slow' velocity responses respectively. Primary and secondary endings differed both with respect to the 'quick' and the 'slow' components of the velocity response. Endings with response characteristics typical of both primary and secondary endings were distinguished as a special group. They have been referred to as endings of intermediate type. Some possible spindle mechanisms responsible for the observed properties of the spindle endings are discussed.

In a previous paper (Lennerstrand and Thoden 1968) an introduction was given to a series of investigations on the dynamic properties of the muscle spindle endings. It was stated that the aim of the work as a whole was to arrive at a quantitative description of the muscle spindle dynamics. For this purpose information on spindle sensitivity to length (*position*) and rate of change of length (*velocity*) would seem sufficient, since no specific spindle sensitivity to acceleration could be detected. The most suitable input signal for this study was found to be length changes of triangular length—time relations.

The results reported in the present paper obtained on passive primary and secondary endings (passive = deprived of fusimotor activation) will serve as the control for the effects of stimulation of single fusimotor fibre to be described later. Therefore only those spindle endings which lasted for a complete test also during

fusimotor activation have been taken into account in the present determination of position and velocity sensitivity

A new method of describing the velocity responses quantitatively was developed, which also seemed to offer a way of grouping the endings in a more functional manner than the conventional differentiation by means of the conduction velocity of their afferent fibres. These findings have led to a discussion on some of the mechanisms by which spindle responses might be generated.

Some points concerning the organization of spindle responses in functionally different types of muscles were also studied. The responses of endings in both flexor and extensor ankle muscles have been examined in order to detect differences in spindle properties between them. Responses of spindles in fast and slow muscle were compared, since Granit and Homma (1959 a b) working on the rabbit, have reported higher static and dynamic sensitivity of spindles in fast muscles than in slow muscles. Some of the results have been reported in a preliminary note (Lennerstrand and Thoden 1967)

Methods

The general procedures and the technique have already been fully described (Lennerstrand and Thoden 1968) New is merely the addition of ankle flexors in the present paper Ankle extensor and flexor muscle spindles were examined i separate experiments. The extensor muscles used were the soleus (Sol.) and the lateral gastrocnemius (LG) muscles. In the first series of flexor experiments the short peroneus muscle (PB) was not separated from the *tibialis anticus (TA) and extensor digitorum longus muscle (EDL) as was done in the later* experiments. The amplitude of stretch, which for extensor muscles was maximally 8 mm, had to be limited to 5 mm in order not t overextend the PB and this amplitude was then used in all the experiments on the flexor muscles. Spindles in the PB showed higher sensitivity to length and rat of change of length than other spindles. This was probably due to the relatively larger extensions of this short muscle, which amounted t about 20 per cent of the resting length in spit of the smaller amplitude of stretch while the corresponding hres for the other flexor and extensor muscles were about 10 per cent. It is assumed, then, that the muscle spindles in the various muscles t be compared are of about the same length, as appears to be supported by anatomical investigations (g Eldred *et al* 1962) although information on this poi t is scant.

Results

Position sensitivity

The slope of the rectilinear relation between the static (position) responses and muscle length (see Matthews 1964) has been termed *position sensitivity* in the present study the method for its assessment has been described previously (Lennerstrand and Thoden 1968)

In Table I mean values and standard deviations of position sensitivity are shown for primary and secondary endings of the different muscles. At first sight secondary endings seemed to have higher position sensitivity than primary endings, but in the present material no statistically significant difference could be detected between the position sensitivities of any of the different groups of spindle endings listed in Table I This also means that such differences between slow and fast muscles as were ob-

Table I Position sensitivity of primary and secondary endings in different hindlimb muscles. Values in impulses/sec/mm

	Primaries		Secondaries	
muscle	mean ± S.D	n	mean ± S.D	n
LG	2.6 ±0.8	7	4.4 ±2.3	
Sol	3.9 ±1.9	16	5.9 ±2.0	10
TA+EDL	4.7 ±2.1	12	7.7 ±4.3	9

served by Granit and Homma (1959 *a*) in the rabbit, have not been seen in the cat.

When comparing our results with those of other authors it must be taken into account that most $f-\Delta L$ (= impulse frequency muscle length) curves in earlier investigations have been plotted from values of discharge rate taken much earlier than the 30 sec after completion of stretching used here. In order to find out if the initial period of constant length influenced the slope of the $f-\Delta L$ curve, two curves obtained from the same ending and referring to values taken at 30 sec and at 0.5 sec after completion of stretching are compared in Fig. 1. One primary (Fig. 1 *A*) and one secondary endings (Fig. 1 *B*) are shown. The 30 sec values were much less dependent on the order in which the values were collected than the 0.5 sec values, whether in a sequence of increasing or decreasing lengths. The small degree of hysteresis seen even in the 30 sec plots was found in all endings studied. It was presumably due to plastic properties of extra- and intrafusal muscle fibres, comparable

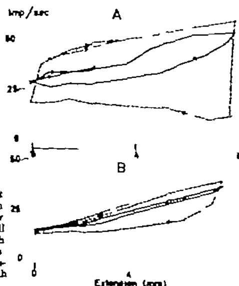


Fig. 1. Impulse frequency values at constant muscle extension plotted against muscle length for one primary ending (*A*) and one secondary ending (*B*). Values obtained at 30 sec (full lines) or at 0.5 sec (broken lines) after each of a succession of small rapid length changes. Arrows indicate the direction of muscle extension, i.e. to length increment or to length decrement.

to those found in single extrafusal fibres in the frog (Buchthal and Kaiser 1951) The main differences in slope between the 0.5 and 30 sec plots occurred during release of stretch. For length increment, however the slopes of the two curves corresponded fairly well. Since in earlier studies, $f-\Delta L$ curves have been plotted almost exclusively for muscle stretch, the results are comparable to those obtained in the present investigations. Most of our results on position sensitivity of spindles in the different muscles of the cat are in agreement with those of previous authors (see *e g* Harvey and Matthews 1961 Matthews 1964 Alnaes, Jansen and Rudjord 1965)

Velocity sensitivity

The velocity sensitivity of an ending can be expressed as the relation between velocity responses and velocity In this study the velocity responses have been acquired from the dynamic responses to square wave changes in velocity produced by length changes of triangular wave form. The velocity responses were calculated by eliminating not only the position response but also some length dependent transients from the dynamically obtained response as shown in a previous paper (Lennerstrand and Thoden 1968) It was found that in the passive secondary ending the velocity response accounted for the whole dynamic response. In the passive primary ending, however the dynamic responses in addition to the velocity response contained some components, which were length dependent. In the paper just quoted, a method was proposed by which the genuine velocity response could be calculated also for the primary endings (see also Fig 3)

1 Primary ending

Velocity responses to length increment The velocity responses of primary endings to length increment and decrement were not equal. This is illustrated in the $f-\Delta L$ diagrams of Fig 2 showing typical responses of a primary ending over the whole range of velocities applied in this work. Velocity responses to length increment will be considered first

Fig 3 shows the dynamic response to length increment in the triangular change of length after subtraction of the position response. The original record is given in Fig. 2 G As shown in a previous paper (Lennerstrand and Thoden 1968) the overshooting initial part of the plot, the so called initial burst response is not related to the input velocity

The initial burst or a possibly occurring length dependent initial i-transient (Lennerstrand and Thoden 1968) should be disregarded and attention paid only to the subsequent portion of the diagram In order to facilitate measurements of the different components of the genuine velocity response, the later portion of the curve was extrapolated to the ordinate for zero extension in Fig. 3 The result will then represent the pure velocity response to stretch. This response can be regarded as consisting of two components 1) A step-like rise from the steady state curve. In Fig 3 the steady state curve has already been subtracted. This quick step occurs at

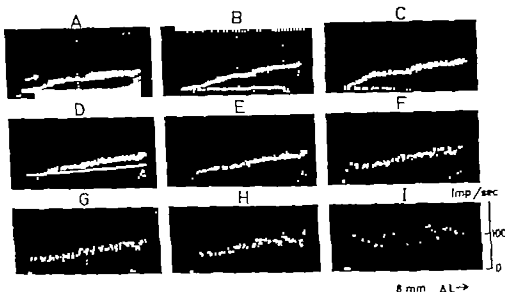


Fig. 2. Responses in $f-\Delta L$ diagrams of primary ending to triangular length changes over the whole range of velocities used in the study. The velocity is in *A* 0.05 mm/sec, *B* 0.1., *C* 0.5, *D* 1, *E* 2, *F* 4, *G* 8, *H* 16 and in *I* 32 mm/sec. Steady state curve represented by full line in *D*. Arrow indicates point movement in time in the loop. Length (ΔL) increases to the right in the pictures.

the initiation of stretch. It has been termed the quick velocity response 2) The second component consists of a progressive increase in impulse frequency steeper than that of the steady state curve. This part has been called the slow velocity response. In Fig 3 it is represented by the slope of the curve in the diagram.

The quick velocity response rapidly rose to a nearly constant value characteristic for the input velocity. This value is given in impulses per sec. The time course of its

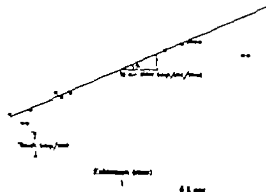


Fig. 3. The dynamic response to 8 mm/sec stretch of the primary ending in Fig 2. Points obtained by subtracting the position response from the values in Fig. 2 *G* hence the steady state curve the zero line of impulse frequency. Full line fitted by eye to the points, disregarding the initial burst response at initiation of stretch. Broken lines are auxiliary lines. The quick velocity response is the intercept on the ordinate between zero impulse frequency and the extrapolated full line. The slow velocity response is the slope of the full line

initial transient was fairly quick although not overshooting. It was generally more rapid for primary than for secondary endings. (For this reason the 'quick' velocity response has been labelled differently for primary and secondary endings: in primary endings it is called the 'quick P' response and in secondary endings the 'quick S' response.) The transient of the 'slow' velocity response, on the other hand, seemed to be of such a slow time course that even at the lowest velocities applied (0.05 mm/sec) no asymptotic value could be reached within the limits of possible extensions. This can be seen in Fig. 2. In the quantitative measurements the 'slow' velocity response must therefore be considered as a continuously increasing quantity added to the 'quick' velocity response as stretching proceeds in time. It has therefore been necessary to give it the dimensions of a slope. Since most of the $f - \Delta L$ curves, from which the measurements have been obtained, have the same length scale but different time scales, the values of the 'slow' velocity responses were most conveniently given in impulses/sec/mm. As will be shown below, the validity of this separation of the two components of the velocity response is based on their different relation to the input velocity.

Quick P' velocity response to length increment. The 'quick P' part of the velocity response generally showed an almost linear relation to the input velocity within the range from 2 to 32 mm/sec. This is illustrated in Fig. 4 A from the same ending as in Fig. 2. At rates below 2 mm/sec no 'quick P' velocity response is observed. A plot of the 'quick P' velocity responses of the whole sample of primary endings

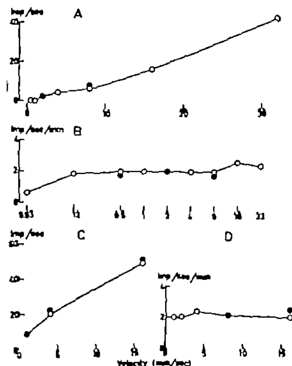


Fig. 4. *A.* Plot against velocity of 'quick P' velocity responses of the primary ending in Fig. 2. Control values represented by open symbols and responses at 2.5 mm increased initial extension marked by filled symbols. *B.* 'Slow' velocity responses of the primary ending in Fig. 2 against velocity in log scale. Symbols as in *A.* *C.* 'Quick P' velocity responses of another primary ending. Responses to length changes of 8 mm amplitude represented by open symbols, filled symbols from length changes of 4 mm amplitude around the same operating point. *D.* 'Slow' velocity responses of the ending in *C* and in same experimental conditions. Symbols as in *C.* *Note.* The curves for relation to velocity of the 'quick' (in *A* and *C*) and the 'slow' components (in *B* and *D*) cannot be directly compared, since the two sets of diagrams have different ordinates. Furthermore the velocity scale is logarithmic in *B* but linear in the other plots.

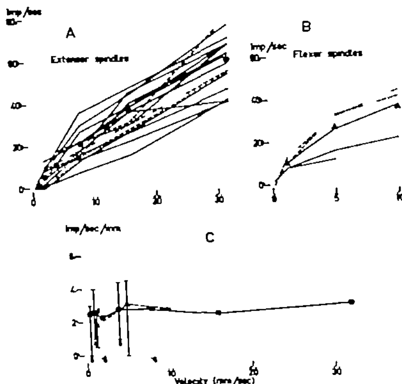


FIG. 5. *A* and *B*. 'Quick' velocity responses against velocity for a population of extensor (*A*) and flexor (*B*) primary endings. Each line represents one ending. In some cases two or more lines partly coincide. Full lines in *A* are endings in Sol. and in *B* endings in TA and EDL muscles. Broken lines in *A* are endings in LG and in *B* endings in PB muscles. Filled symbols mark average values for each population. *C*. Plot against velocity of average values of slow velocity responses for primary endings in extensor (circles) and flexor muscles (triangles). Vertical bars indicate total range of values. Figures at bars are the number of observations in the average value for flexor endings marked on top of bars and for extensor endings below bars.

against the velocity of stretch is shown in Fig. 5 *A* for extensor endings and in Fig. 5 *B* for flexor endings. The average values in this plot deviated from linearity.

Some flexor primaries of the PB muscle (interrupted lines in Fig. 5 *B*) had larger quick P velocity responses than seen in the extensor endings. This may be due to the fact that the PB spindles were exposed to relatively higher rates of length changes than were the spindles in the other muscles as explained in Methods.

Slow velocity response to length increment. In all the primary endings tested at the lowest velocities used in this study, a slow velocity response could be recognized. Fig. 2 *A* thus shows the presence of slow responses even at a rate of 0.03 mm sec. However, in sharp contrast to the quick response, the slow response did not increase with increasing velocity above 0.1 mm sec (or sometimes 0.5 mm sec). At this level the slow response became saturated as it were, and further increase of the

velocity did not cause any significant increase in the 'slow' velocity response (Fig 4 *B*) This difference in responsiveness of the quick and 'slow' components of the velocity response makes it necessary to treat them individually

Flexor and extensor endings showed almost identical average values of slow velocity response as can be seen in Fig 5 *C* where the bars represent the ranges of the observed values. The number given above or below the bars indicate the number of observations underlying each average value.

Responses to length decrement During release of stretch primary endings have a tendency to cease firing (Cooper 1961 Harvey and Matthews 1961 Bessou and Laporte 1962 Matthews 1963 Alnaes, Jansen and Rudjord 1965) a fact which prevents assessments of any velocity response during length decrement. The cessation must be due to the velocity response to muscle shortening in each moment being equal to the position response. In terms of the underlying generator potentials (Katz 1950 Ottoson and Shepherd 1965) it is possible that the change belonging to the velocity response is larger than that of the position response. It was also found that in most primary endings the velocity response to length decrement was larger than that to length increment the area of the dynamic $f-\Delta L$ diagram below the steady state curve is larger than the area above the steady state curve at all velocities represented in Fig 2. Since most endings stopped firing to muscle release at absolute values of velocity above 0.5 mm/sec (see Fig 2) no measurements on velocity responses to length decrement were performed. Some primary endings were even more sensitive to muscle shortening and ceased firing on shortening even at the slowest rates used (0 03 mm/sec) while other less sensitive endings could fire impulses during muscle shortening at velocities higher than 0.5 mm/sec.

The latter type of endings, constituting 10—20 per cent of the total number of primary endings, resembled secondary endings in their responses to length decrement, although the conduction velocity of their afferent fibres was in the range of the primary endings. They will be dealt with in section 5

2. *Secondary endings*

Two features of secondary endings which distinguish them from primary endings are their small dynamic responses to stretch (Cooper 1961 Harvey and Matthews 1961 Bessou and Laporte 1962 Matthews 1963) and the firing also during release of stretch (Cooper 1961 Harvey and Matthews 1961 Bessou and Laporte 1962) Velocity responses of secondary endings should be possible to measure also to length decrement. To the earlier findings can be added the new result that most of the secondary endings do not possess the 'quick P' velocity response, characterized in primary endings by the very rapid shift in impulse frequency *at the instant* of the step change in velocity

The $f-\Delta L$ diagrams of Fig 6, from a secondary ending, shows typical samples of the responses to velocities between 0.5 and 32 mm sec. No initial burst is obscuring the early part (*f* Lennerstrand and Thoden 1968) The rate of firing during the release follows a curve roughly parallel to that during stretching, al-

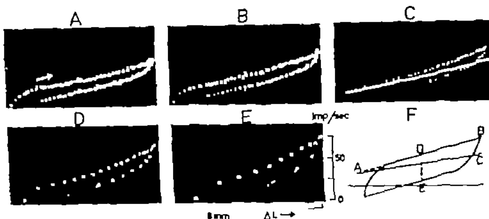


Fig. 6 *A—E*. Responses in $f-\Delta L$ diagrams of secondary ending to 'triangular' length changes of different velocities, in *A* 0.5 mm/sec, *B* 4, *C* 8, *D* 16 and *E* 3 mm/sec. Markings as in Fig. 2. *F* Diagrammatic drawing of the response to 'triangular' length changes of the secondary ending to show measurements of 'quick S' and of 'slow' velocity responses. The parts of the diagrams above zero impulses frequency represented by thick lines. The line A—C is the steady state curve. Broken lines serve as auxiliary lines. The 'quick S' velocity response is the distance D—E. The 'slow' velocity response is the difference in slopes between lines A—B and A—C. Note quick S' response comprises quick velocity responses both to length increment and to length decrement. For explanation see text.

though shifted downwards to lower impulse frequencies. At minimal or maximal extension, a change in direction of the movement does not elicit any rapid shift in the impulse frequency that would signify the 'quick P' velocity response. Such velocity responses have been observed in but 3 of 25 secondary endings and in these cases they could be detected only at the three highest velocities.

The width of the $f-\Delta L$ curves to triangular extensions in Fig. 6 is seen to increase with velocity. The component of the velocity response responsible for this change probably develops along a time course which is slower than that of the quick response of the primary ending but considerably more rapid than that of the slow response. As mentioned above this velocity response of the secondary endings has been termed 'quick S' velocity response. In Fig. 6 *F* the quick S velocity response is the distance D—E. It is measured as the width of the $f-\Delta L$ loop at half maximal extension after subtracting the hysteresis of the steady state curve and is expressed in imp/sec. This method does not give separate information about the quick S' velocity responses to length increment and decrement. There are reasons to believe that the quick S response to length decrement is slightly different from that to length increment (see Appendix).

'Slow' velocity responses were recognized also in the responses of secondary endings. In the schematic drawing of Fig. 6 *F* the slow response is shown as the difference in slope between the line A—B of the $f-\Delta L$ diagram and the steady state curve A—C. It is measured in imp/sec/mm.

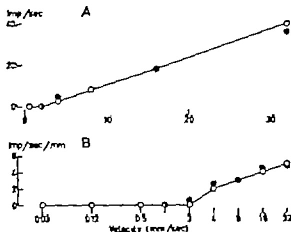


Fig 7 A 'Quick S' velocity responses of a secondary ending against velocity Responses to length changes at original (open symbols) and at 2.5 mm increased initial extension (filled symbols) B. 'Slow' velocity responses of the ending in A against velocity in log scale. Changes in initial extension as in A and indicated by the same symbols. Note: Because of differences in ordinates and velocity scales the curves in the two diagrams cannot be directly compared.

The quick S' velocity response In the secondary ending represented by the plot in Fig 7 A a quick S' response appeared in the $f-\Delta L$ diagram at 2 mm sec and at higher velocities it increased linearly with velocity The ending in Fig 6, on the other hand, showed a quick S velocity response already at 0.5 mm sec but this response did not increase until the velocity reached values above 2 mm sec. Many secondary endings behaved in the same way at low velocities as can be seen in Fig 8 A and B and for these endings the curves of quick S velocity responses against velocity showed a large step from zero, at steady state to the fairly high values at 0.5 mm sec. No satisfactory explanation for this behaviour has been reached. The average values of quick S' velocity responses in extensor (Fig 8 A) and flexor

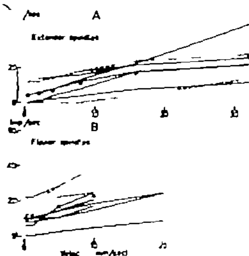


Fig. 8

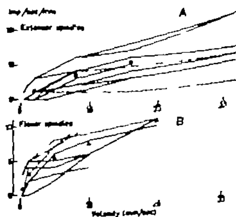


Fig 9

Fig. 8. 'Quick S' velocity responses against velocity for a population of extensor (A) and flexor (B) secondary endings. Lines and symbols as in Fig. 5

Fig 9 'Slow' velocity responses against velocity for a population of extensor (A) and flexor (B) secondary endings. Lines and symbols as in Fig 5

(Fig 8 *B*) secondaries were linearly related to velocity only in the lower velocity range. As a rule the quick responses of the secondary endings were smaller than those of primary endings.

The slow velocity responses of a secondary ending are shown in a plot against velocity on a log scale in Fig. 7 *B* A 'slow' velocity response was not observed at velocities below 1—2 mm/sec. Above this threshold velocity the slow component of this ending was linearly related to log velocity However the average values of extensor and flexor endings were not linearly related to log velocity over the whole range of velocities In Fig 9 the 'slow' responses of the populations of extensor (Fig. 9 *A*) and flexor (Fig. 9 *B*) secondaries have been plotted against velocity on a linear scale.

Velocity responses in flexor and extensor endings. The quick S' velocity responses were about the same for flexor and extensor secondary endings (Fig 8 *A* and *B*) The 'slow' velocity responses of some flexor endings in the PB muscles were appreciably larger than those of extensor endings (Fig. 9 *A* and *B*) An explanation of this difference has already been suggested (see Methods) For the rest of the flexor secondaries the slow velocity responses fell within the range of the values of extensor secondaries.

3. *Velocity responses of spindle endings in fast and slow muscles*

Granit and Homma (1959 *b*) working on the rabbit spindles, found the responses to the phasic part of muscle stretch to be higher in spindles in fast muscles than in those of slow ones. In the cat no corresponding difference was found for velocity responses of primary and secondary endings in the fast (LG) or the slow (Sol.) muscles. This can be seen from Fig. 5 *A* and from Figs. 8 *A* and 9 *A* in which the full lines represent endings in the Sol. and the broken lines are from endings in the LG muscle.

4. *Varying the initial muscle tension and the amplitude of the length change*

These variables were introduced in order to find out whether passively induced changes in intrafusal tension by any chance influenced the quick and 'slow' parts of the velocity responses. If so the latter cannot be regarded as expressions of a genuine velocity sensitivity It was found that 1) altering the operating point of the length change to a maximum 5 mm larger extensions, or 2) decreasing the amplitude of the length change to half the original value around the same operating point, did not induce any change in the quick and 'slow' velocity response, neither in primary nor in secondary endings. This is illustrated in Figs. 4 and 7 In which the results, marked by filled symbols, were obtained at an increased initial muscle extension of 25 mm (Fig 4 *A* and *B* Fig 7 *A* and *B*) or from a halving of the amplitude to 4 mm (Fig 4 *C* and *D*) The open symbols are the control values. However for the primaries the results refer only to responses obtained during length increment Altering the initial length sometimes changed selectively the response of some primary endings to length decrement (see below)

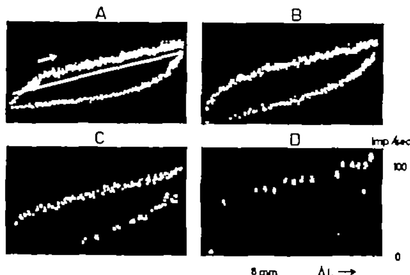


Fig 10 Responses i $f-\Delta L$ diagrams of an ending of intermediate type to 'triangular' length changes of different locities in *A* 0.5 mm/sec, *B* 2, *C* 8 and in *D* 32 mm/sec. Markings as in Fig 2.

5. *An intermediate type of response*

As mentioned above some endings with afferent conduction velocities even in the range of primaries, above 80 m/sec, fired impulses also during muscle shortening at relatively high velocities in a manner typical of the secondary endings. Their veloci ty responses to length increment, however was of the type characteristic for primary endings. These endings ha e accordingly been denoted as endings of intermediate or intermediate endings.

The typical responses of an intermediate ending to 'triangular length changes of elocities between 0.5 and 32 mm sec are depicted in Fig 10 'Quick P' elocity responses and slow elocity responses to length increment fall within the range of values obtained for ordinary primary endings. The change with elocity in the slow velocity response to length decrement is similar to that of the same response in secondary endings. The intermediate ending of Fig 10 seems to have a quick P velocity response also to length decrement. However the magnitude of this response is reduced in length changes of equal elocity but of increased initial muscle extension (*cf* F g 7 *B* in th paper of Lennerstrand and Thoden 1968) The impulse frequency shift at ma mal extension is therefore unlikely to be a genuine quick P velocity response. Because of the assumed combination of response components from primary and secondary endings in the intermediate type of ending, a significant ex pression for a quick S elocity response to length decrement is hard to obtain. In general this velocity response seemed to be larger in intermediate than in secondary endings intermediate endings ceased firing at a lower elocity of length decrement than the secondary endings although the two types of endings had about the same position responses.

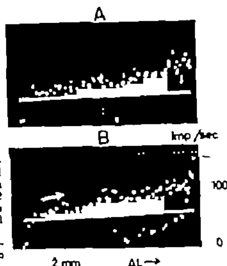


Fig. 11 Initiation of impulse firing to length decrement in primary ending, which at low initial muscle extension (A) is silent during release in triangular extensions of 2 mm amplitude and 2 mm/sec elocity The ending starts to fire impulses to shortening t the same elocity when the initial extension in *B* is increased about 2 mm. Note I contrast to the usual behaviour the position sensitivity of this ending is increased by the shift in operating point.

The properties of intermediate endings were more *frequently represented in the* group of endings ith afferent conduction velocities between 60 and 80 m sec. This group of intermediate conduction rates also contained endings both of the type described as typical for primary endings and of the type characteristic for secondary endings. If only the response characteristics and not conduction rates are used as the means of classification of the spindle endings, our material on extensors contains 27 endings of the primary type 19 of the secondary type and 10 endings of the intermediate type.

A few of the primary endings, which became silent even at a low velocity of shortening, could at the same velocity discharge impulses during length decrement, if th initial muscle extension was increased. The discharge pattern to shortening was in these cases indistinguishable from that of an intermediate ending. This effect of altering the initial muscle length is illustrated in Fig 11 It can be seen that the velocity response to muscle shortening became smaller than it was at the original initial length. The velocity response to length increment however was unchanged. Endings which already at a low initial extension had response characteristics of the intermediate type maintained this behaviour at increased initial extension. However their elocity responses to length decrement were also reduced by the increased initial extension.

Discussion

The data presented in this paper on the dynamic analysis of muscle spindle endings deprived of fusimotor activation are intended to be used as the background for further studies on the dynamics of spindles activated by their fusimotor nerve fibres. Ultimately nalogue simulation of the spindle mechanisms will be attempted. In the

Appendix are presented the mathematical expressions of the position and velocity responses of the passive spindle derived from the results of this paper. Some of the servo technical aspects of the results will, however, not be discussed until the simulation of the whole spindle machinery is presented. The present discussion will therefore be confined to the points to follow.

Velocity response. The exact mechanisms in the spindle, underlying the different components of the velocity responses, are not known. It seems, however, generally held that the dynamic properties of muscle spindles depend mainly on the interaction between elastic and viscous elements in the intrafusal fibres and possibly to some extent also in the extrafusal muscle (Lippold, Nicholls and Redfearn 1960, for further references see Matthews 1964), although it cannot be excluded that part of the derivating property of muscle spindles may depend also on the mechanisms by which the deformation of the receptor membrane is converted into receptor potentials, as discussed by Katz (1950). On the other hand the relation between the receptor potentials or current and the afferent discharge rate seems to be a direct one (Edwards 1955, Lippold, Nicholls and Redfearn 1960, Ottoson and Shepherd 1965).

Differences in dynamic properties between passive primary and secondary endings have been reported earlier by Harvey and Matthews (1961) and Matthews (1963) and are confirmed in the present study. It was found in this paper that primary endings had larger quick responses than secondary endings and that the relations to velocity of the slow velocity responses were quite different in the two types of endings. This would indicate that the activity of primary and secondary endings originated from separate spindle structures with different mechanical properties. It has been proposed that the transients in dynamic responses of primary endings mainly reflect events in nuclear bag fibres while the much smaller transients in the dynamic responses of secondary endings depend on the mechanics of nuclear chain fibres (Harvey and Matthews 1961, see also Matthews 1964). This idea was originally based on the anatomical findings that the terminals of primary endings are distributed to both nuclear chain and bag fibres, while the terminals of secondary endings lie almost exclusively on nuclear chain fibre (Boyd 1962, Barker 1962). It is now known from the direct observations by Smith (1966), confirmed by Boyd (1966 a, b) and Diete-Spiff (1966), that thick intrafusal fibres (presumably nuclear bag fibres) have larger viscous damping than thin intrafusal fibres (probably nuclear chain fibres). This would give the nuclear bag endings larger dynamic sensitivity than the nuclear chain endings, which has also been observed in this and earlier studies.

An observation, which might be of relevance in this context, is that the contractile properties of nuclear bag fibres resemble those of slow muscle fibres in the amphibian (Smith 1966, Boyd 1966 a, b, Diete-Spiff 1966), while the nuclear chain fibres in these respects are more like twitch fibres (Smith 1966, Boyd 1966 a, b). Perhaps the differences between primary and secondary endings with respect to quick and slow velocity responses are depending on differences in mechanical properties be-

tween nuclear bag and nuclear chain fibres, if they are slow and twitch fibres respecti ely

The increased dynamic sensitivity of many primary endings during release of stretch must probably also be given a mechanical explanation.

Alternatively th diminution of impulse discharge during muscle shortening could depend on so called post tetanic depression. However the maximal impulse fre quency of the endings seems much too low to cause the profund depression seen in some endings. Furthermore, such depression is not seen in all primary endings, the exceptions being the endings of the so called intermediate type It is also absent in endings relaxed by graded reduction of intrafusal contraction although the firing can reach the same maximal discharge rate as in response to stretch (Andersson. Lennerstrand and Thoden, unpublished observation) For these reasons the hy pothesis of post-tetanic depression seems unlikely

A mechanical explanation of the beha iour of primary endings during release of stretch would imply that the intrafusal tension would fall below the threshold for impulse generation during the release. Simple dry friction in the spindle elements can be excluded, since it would affect to the same degree the steady state responses to muscle shortening A sufficient fall in intrafusal tension would probably be best explained by assuming that the viscous forces for some reason are larger during release than d ring stretch in the nuclear bag fibre. This view is fa oured by some preliminary results on the length-tension relations in single slow muscle fibres of the toad obtained recently by Lännergren (personal communication) the tension changes ppeared to show similar dependence of the direction of the length change as the impulse frequency responses of the primary endings. In nuclear chain fibres both phases of the movement seem to generate almost equal viscous forces as re vealed by the impulse frequency responses of secondary endings. In this respect nuclear chain fibres would resemble amphibian twitch fibres (Kaiser and Buchthal 1951)

Intermediat ending Using the criteria of afferent conduction velocity most spindle endings that show responses of 'intermediat type were grouped as primary endings. The classification is further supported by the fact that intermediate endings can be activated by dynamic fusimotor fibres (Lennerstrand and Thoden, unpublished observations) which are known to effect almost exclusively primary endings (Appelberg, Bessou and Laporte 1966) The intermediate endings showed characteristics typical of primary endings in their responses to length increment and of secondary endings in their responses to length decrement.

Although the possibility exists that the spindle responses of intermediate type might originate from terminals on, for instance, the myotube regions of the nuclear bag intrafusal fibre or even on a separate intrafusal fibre like the one of intermediate type described by Barker (1962) we would like to suggest another inter pretation. It was found in the present study that the dynamic response of inter mediate endings to length decrement was affected by an increase in the initial muscle extension but not the response to length increment. This can be taken to

indicate that the impulse firing of 'intermediate' endings during stretch and release depend on separate spindle mechanisms. Retaining the hypothesis that the responses of typical primary and secondary endings derive from nuclear bag and chain elements respectively one explanation for the mixture of both responses represented by intermediate endings would be that there are different trigger zones for impulse generation in the afferent terminals from the bag and the chain elements and that the zones participate alternatively to compose the response of intermediate type. A similar hypothesis was first brought forward by Crowe and Matthews (1964) in order to explain the results obtained on stretching primary endings during stimulation of single fusimotor fibre at a constant rate. They suggested that the impulse frequency of the afferent fibre would at any time, correspond to that receptor site giving the highest discharge rate and thus acting as the pace maker. These concepts have, for instance, been found to be valied for tactile receptor units in the skin of toad, cat and monkey (Lindblom 1958 Lindblom and Tapper 1966)

One would assume from our present view of the anatomy of the muscle spindle that the responses of intermediate type would be particularly common in the primary ending. It seems therefore strange that the typical response of primary endings does not contain any component which appears to be directly related to their nuclear chain terminals. It seems unlikely that the majority of the primary endings in this study would be without nuclear chain terminals, since such primaries are rare (Barker 1962). Instead it could be that the excitation of the nuclear chain component of the primary endings on mechanical or electrical grounds usually has been below threshold for impulse generation under the present experimental conditions. This idea is supported by the finding that an increase in the initial muscle extension in some primary endings could induce an impulse frequency response to shortening, hich resembled that of secondary endings (Fig. 11 B). It can be taken to mean t in these cases the threshold was reached for impulse generation in the nuclear ain component

dentification of pindle ending from $f-\Delta L$ diagrams. The method of identifying the different spindle endings functionally from their $f-\Delta L$ diagrams to 'triangular' movements has been found to be a useful tool supplementing the conventional one based on afferent conduction velocities. In this way also the group of endings with fibres in the intermediate range of conduction velocities between 60 and 80 m/sec could be reliably classified as primary or secondary endings. Above all, the existence of a nuclear bag component in the $f-\Delta L$ diagram is decisive for the functional grouping of the endings, since from the histological findings such components are expected to be present in the responses of primary endings but absent in secondary endings. On these grounds all the endings in this study subjected to length changes over a reasonably large range of velocities, could in fact be grouped into primaries and secondaries on the basis of their $f-\Delta L$ diagrams.

Functional identification of spindle endings making use of the differences in vibrational sensitivity between primary and secondary endings (Bianconi and Van der Meulen 1962, Brown, Engberg and Matthews 1967) seems also to be highly

selective. The difference in vibration sensitivity probably reflects the same difference in mechanical properties between nuclear bag and nuclear chain fibres as those underlying our $f-\Delta L$ diagrams the responses to vibration should therefore also be regarded as a useful supplementary check on every functional hypothesis presented in a model of muscle spindle dynamics.

Appendix

by

Bengt Andersson

(Division of Automatic Control Royal Institute of Technology Stockholm 70, Sweden)

The equation given below is intended to summarise the present results in mathematical form. It is only first pproximation, but should nevertheless be of interest in attempts to simulate the spindle in model. However no such ttempt to interpret the results in physical terms will be made at present

With the definitions given below the ariations in impulse frequency as first approximation, is given by

$$f - f_0 = k\,\Delta L \qquad + g\,\frac{}{1+T}\,\Delta L \quad + h\,\Delta L \qquad (1)$$

= 'position term + 'quick term + 'slow term

where

f instantaneous impulse frequency
f_0 steady impulse frequency to 'zero extension
k position sensitivity
ΔL length change
Laplace operator
g, h } functions of velocity
T time constant

If g had been constant and k equal to zero eq (1) would hav been linear and simila to that for so called 'lead network that is, differentiating over limited frequency

Position' term.

The position sensitivity k, has already been explained in connection with the experimental results.

Quick term.

The values of the time constant, T seem to be rather different for the primary and the secondary ending thus necessitating slightly different treatment of the quick P' term primary) and the 'quick S' term (secondary)

Quick P' term I this case the time constant, T is considered to be ery short ($\ll 1$)

If

$T \ll 1$ sek

then $g\,\frac{}{1+T}\,\Delta L \approx g$

here

velocity (ΔL m)

The quantity g is represented as a function of elocity in Fig. 4 A 4 C 5 A and 5 B It must be observed that the 'quick P' term can be measured only for muscle stretch.

indicate that the impulse firing of 'intermediate' endings *during stretch and release* depend on separate spindle mechanisms. Retaining the hypothesis that the responses of typical primary and secondary *endings* derive from nuclear bag and chain elements respectively one explanation for the *mixture* of both responses represented by intermediate endings would be that there are different trigger zones for impulse generation in the afferent terminals from the bag and the chain elements and that the zones participate alternatively to compose the response of intermediate type. A similar hypothesis was first brought forward by Crowe and Matthews (1964) in order to explain the results obtained on stretching primary endings during stimulation of single fusimotor fibre at a constant rate. They suggested that the impulse frequency of the afferent fibre would at any time, correspond to that receptor site giving the highest discharge rate and thus acting as the pace maker. These concepts have for instance, been found to be valid for tactile receptor units in the skin of toad cat and monkey (Lindblom 1958, Lindblom and Tapper 1966).

One would assume from our present view of the anatomy of the muscle spindle that the responses of intermediate type would be particularly common in the primary ending. It seems therefore strange that the typical response of primary endings does not contain any component which appears to be directly related to their nuclear chain terminals. It seems unlikely that the majority of the primary endings in this study would be without nuclear chain terminals, since such primaries are rare (Barker 1962). Instead it could be that the excitation of the nuclear chain component of the primary endings on mechanical or electrical grounds usually has been below threshold for impulse generation under the present experimental conditions. This idea is supported by the finding that an increase in the initial muscle extension in some primary endings could induce an impulse frequency response to shortening, which resembled that of secondary endings (Fig. 11 B). It can be taken to mean that in these cases the threshold was reached for impulse generation in the nuclear chain component.

Identification of spindle endings from f – ΔL diagrams. The method of identifying the different spindle endings functionally from their f – ΔL diagrams to triangular movements has been found to be a useful tool supplementing the conventional one based on afferent conduction velocities. In this way also the group of endings with fibres in the intermediate range of conduction velocities between 60 and 80 m/sec could be reliably classified as primary or secondary endings. Above all, the existence of a nuclear bag component in the f – ΔL diagram is decisive for the functional grouping of the endings, since from the histological findings such components are expected to be present in the responses of primary endings but absent in secondary endings. On these grounds all the endings in this study subjected to length changes over a reasonably large range of velocities, could in fact be grouped into primaries and secondaries on the basis of their f – ΔL diagrams.

Functional identification of spindle endings making use of the differences in vibrational sensitivity between primary and secondary endings (Bianconi and Van der Meulen 1962, Brown, Engberg and Matthews 1967) seems also to be highly

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Acta physiol. scand. 1968. 73 300—304

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Thermography as an Aid in Hibernation Research

By

P I Brånemark and B. W Johansson

Received 20 November 1967

Abstract

Brånemark, P I and B. W Johansson *Thermography as an aid in hibernation research* Acta physiol. scand. 1968. 73 300—304

The technique of thermography is presented as a aid in hibernation research work as well as in other experiments in which the skin circulation is of interest.

Hibernators of which the hedgehog is one example in Sweden, are characterized by the ability to spend the winter season at a body temperature a few degrees above freezing-point and to arise spontaneously to a "normal" body temperature. During this arousal the fore part of the animal including the brown fat (Johansson 1959) heart and brain has a higher temperature than the hind part. During hibernation the animal is very sensitive to tactile stimuli a needle puncture for the injection of a drug is thus enough to initiate the arousal process. Because of this, techniques have been developed for chronic implantation of catheters in the vessels (see Johansson 1963 for review) for injection purposes and for continuous recording of blood pressure and other physiological parameters. It follows that much would be gained by being able to estimate the circulatory state of the animal without touching it at all, or only slightly

Thermography seems to be helpful in this respect. This technique measures and delineates the temperature differences on the surface of a body Although the infrared emission emanates only from the surface area down to a depth of 100 μ the thermogram may permit an estimate of the state of the underlying tissues in view of the close circulatory connections existing between these tissue layers.

Material and methods

Four naturally hibernating hedgehogs (*Erinaceus europaeus*) were used in the month of February 1963 Arousal was induced by placing the animals in room of bout 20 C. Body temperature was measured with thermocouple and electrocardiograms ere recorded as de-

Manufactured by ICI, Macclesfield, England.
Manufactured by AGA AB Stockholm, Sweden.

scribed earlier (Biörck and Johansson 1955) though using a direct-writing ink jet electrocardiograph (Elema). One animal was given Inderal, 1-isopropylamino-3-(1-naphtyl oxy)-2-propanol hydrochloride. This substance which is an inhibitor of the β-adrenergic receptors has been found to prolong the arousal procedure and partially inhibit the differential warming (Johansson 1965).

During arousal the infrared emission was recorded with an AGA Thermovision unit in an air-conditioned room at 19—20 C at intervals of 15 to 30 min until the body temperature was about 30 C. The animals were stretched out from the curled position and placed with the entral part upwards, a more detailed thermogram was obtained if the entral surface was used instead of the dorsal, with its quills and thick skin. The first thermogram was recorded as soon as possible after the animal had been removed from its hibernation nest.

The Thermovision unit works according to a scanning principle utilizing the body's normal infrared emission in the spectral region of 2—20 μ. The object is searched point by point via a system of mirrors and prisms, which directs the emission to an indium-antimonide detector cooled by liquid nitrogen. The optic signals is then transformed into an electronic impulse, which is amplified and modified to give a picture on an oscilloscope screen. This visual picture can be recorded photographically. An objective evaluation of different emission levels is obtained by isotherms, which can be set at various levels and introduced in the infrared image on the screen. This enables an immediate and objective numerical analysis of the emission patterns.

Results and comments

Fig. 1—4 show representative thermograms from the hedgehogs during arousal.

The white areas indicate a relatively higher temperature when compared with the black ones, except in Fig. 1 b which shows an inverted picture, the black area indicating the highest temperature. The absolute temperature difference in degrees centigrade between the white and black areas is indicated by the heavy white bar at the lower part of the figures, the distance between two small white bars being one degree centigrade.

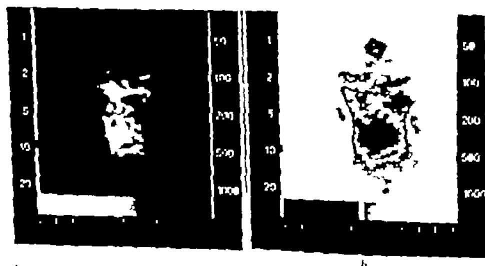


Fig. 1. Emission patterns at start of rewarming: maximum over abdomen and low thoracic region. Heart rate 20 beats/min.

Inverted picture with isotherms to show the maximum emission 1 1 hr later. Heart rate ... beats/min.

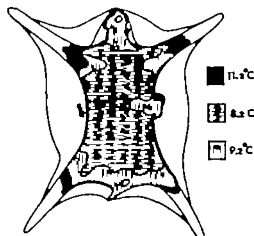


Fig 2. Temperature distribution over entral aspect of hedgehog at start of rewarming obtained by superposition of series of isotherm recordings

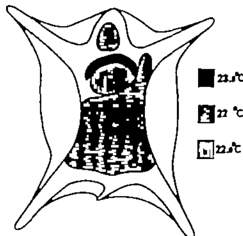


Fig. 3 Temperature distribution over entral aspect of hedgehog 2 1/2 hrs after start of rewarming Not that an area with maximum temperature is situated over the thoracic region over the heart and large essels.

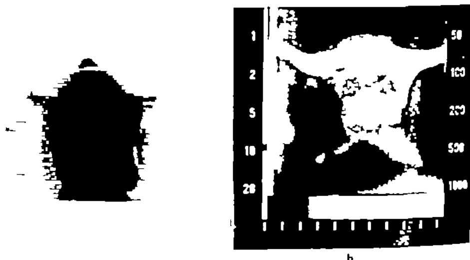


Fig 4 Emission patterns) 1 hr and b) 5 hrs after start of rewarming This hedgehog as given 0 9 mg/kg body weigh of Inderal intraperitoneally Not that the temperature gradient between the thora and the peripheral part of the body is much smaller ( ) than in b) when the Inderal ffec has disappeared See further Fig. 5

Fig 1—3 clearly show th differential warming between the fore and hind part of the hedgehog d ring arousal w th a higher temperature in the thoracic region, including the axillae, where the brown fat is situated. It has earlier been shown that Inderal prolongs the arousal procedure and partially inhibits the differential warming (Johansson 1965) The effect of Inderal is clearly seen in Fig 4 with a much smal-

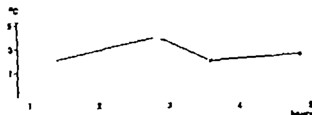


Fig. 5. Temperature difference in degrees centigrade between the central thoracic area and the periphery of the body at different time intervals after initiation of arousal in hedgehog pretreated with Inderal ——— (see further Fig 4 and the text) and in hedgehog given no pretreatment, ———

ler temperature gradient one hr after than five hrs after the Inderal injection when the effect has vanished. The effect of Inderal on the differential warming is graphically illustrated in Fig 5

The present studies were presented in a preliminary form at the Third International Symposium on Natural Mammalian Hibernation in Toronto (Johansson 1965) At the same symposium Hayward and Lyman (1965) independently presented thermograms from bats. The present techniqu has also been used by Hayward and Ball (1966) in studies confirming the brown adipose tissue to be a potent energy source during arousal. Clinicians ha e also become interested n this technique and a conference on this subject was held in New York in 1963 (Gershon-Cohen and Barnes 1964) and in Strasbourg in 1966 (Brånemark 1967)

An advantage of this technique in hibernation research is that one aspect of the circulation can be observed without touching the animals. This makes it possible to study various stimuli's influence on the peripheral circulation. It must be remembered, however that the thermogram records temperature differences only n the superficial layer of the skin it follows that temperature differences in more centrally situated tissues must spread to the surface area before they can be detected by thermography

Furthermore hibernators are curled p during hibernation and in some animals, e.g the hedgehog their position is not fa vorable for thermographical recordings. In other hibernators with a thinner skin and a sparse fur this is probably only a minor drawback.

The purpose of this communication is to point out the possibilities of using thermography in studies of skin circulation in research work, whether concerning hibernators, hypothermic animals, the effects of drugs on animals or in clinical work.

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Acta physiol. scand. 1968. 73 305—310

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Fecal Sterols in Infants

Bile Acids and Steroids 194

By

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Abstract

Gustafsson J A. and B. Werner. *Fecal sterols in infants* Acta physiol. scand. 1968 73 305—310

The predominant sterols in feces of 31 infants have been studied by gas-liquid chromatography and gas chromatography-mass spectrometry Cholesterol, β-sitosterol, and campesterol were the predominant sterols in feces of infants under one year of age. At about the age of 1 year coprostanol began to appear in feces After this time the predominant fecal sterols were coprostanol, cholesterol, campesterol, 24α-ethylcoprostanol, and β-sitosterol. The sterol pattern of feces of infants from 5 months of age up to the highest age investigated (1 year and 9 months) however was characterized by considerable individual variation.

Cholesterol* is converted to coprostanol by intestinal bacteria (Dam 1934 Coleman and Baumann 1957b, Rosenfeld *et al* 1954) Thus coprostanol is absent from the feces of germfree rats (Evrard *et al.* 1964 1965, Gustafsson *et al.* 1966) Coleman and Baumann (1957) have studied the influence of age upon the pattern of fecal sterols in rat. They found that the feces of rat weanlings contain coprostanol. It is not known, however when human beings begin to excrete coprostanol. It was therefore of interest to study the sterol composition of feces from infants, with special reference to the conversion of cholesterol to coprostanol. This could help to gain knowledge of the time when microorganisms are introduced and established in the intestine.

Materials and methods

Collection of f Feces were collected from 31 healthy infants on ordinary infant diet, *e.g.* breast milk or formulas based on cow milk during the first weeks, later with addition of mixed food, *e.g.* fruit, vegetables, meat The feces were collected in polythene bags and immediately stored at −20° C.

* The following systematic names are given to steroids referred to in this communication by trivial names cholesterol, cholest-5-en-3β-ol coprostanol, 5β-cholestan-3β-ol cholestanol, 5α-cholestan-3β-ol lathosterol, 5α-cholest-7-en-3β-ol β-sitosterol, 24α-ethyl-cholest-5-en-3β-ol campesterol 24α-methyl-cholest-5-en-3β-ol 24α-ethylcoprostanol, 24α-ethyl-5β-cholestan-3β-ol.

Analysis of fecal sterols. About 100 mg of feces were transferred to glass tubes and 2 ml of 10 M NaOH was added. The mixture was stirred with a glass rod at 70° until the fecal lumps had disintegrated and a uniform suspension was obtained. Then 2 ml of ethanol, and 2 ml of distilled water was added. After 45 min at 70° the hydrolysate was acidified with 3 M HCl and was extracted with 9 ml of petroleum ether. The petroleum ether phase was washed with 70 % ethanol in water until neutral, concentrated under reduced pressure and transferred to a test tube, where it was taken to dryness under a stream of nitrogen.

Reference compounds. Cholesterol was obtained from Apotekarnes Droghandel AB. Coprostanol was prepared microbiologically from cholesterol by intestinal microorganisms *in vitro* (Snog-Kjær *et al.* 1956) and purified by recycling chromatography on methylated Sephadex (Nyström and Sjövall, to be published). β-Sitosterol and campesterol were obtained from commercial β-sitosterol (Fluka AG, Buchs, Switzerland) also by recycling chromatography on methylated Sephadex. The compounds used in the quantitative determinations were finally recrystallized to constant melting point.

Gas-liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS). The neutral fecal steroids were analyzed by gas-liquid chromatography on an SE-30 column (2 m×4 mm, 1 % SE-30 on acid washed silanized Gas Chrom P). The sterols were analyzed before and after preparation of their trimethylsilyl (TMS) ethers. The conditions used in the GLC analyses were generally as follows: column temperature 220°, flash heater temperature 280°, detector temperature 240°, argon inlet pressure 1.0 kg/cm².

In order to ascertain the identity of the isolated sterols, some representative samples were analyzed as TMS ethers on the LKB 9000 Gas Chromatograph-Mass Spectrometer (LKB-Produkter AB, Stockholm-Bromma 1, Sweden). The column used was a 2.5 % SE-30 column and the conditions were as follows: column temperature 238°, flash heater temperature 280°, molecule separator temperature 245° and ion source temperature 270°. The energy of the bombarding electrons was 22.5 eV and the ionizing current 60 μA.

Preparation of TMS ethers. The TMS ethers (Wells and Makita 1962) were prepared as follows: to the sample was added 0.50 ml of pyridine, 0.20 ml of hexamethyldisilazane and 0.10 ml of trimethylchlorosilane. After 1 hr the reaction mixture was dried under a stream of nitrogen and the residue extracted with hexane and analyzed within one day.

Relative amounts of fecal sterols. All measurements of peak areas were made by planimetry. The calculations were made on the TMS ether derivatives of the fecal sterols. Pure reference samples of coprostanol, cholesterol and β-sitosterol were investigated as TMS ethers and were all found to give linear and identical response.

Results

Identification of fecal sterols. Table I shows the sterols identified in the feces. The GC-MS analyses revealed a completely pure cholesterol peak in all samples investigated by mass spectrometry.

Fig. 1 shows the gas chromatographic analyses of the TMS ethers of the fecal sterols of subjects 26 and 24 (see Table II). Mass spectrometric analysis revealed the identity of the following peaks: A = coprostanyl TMS ether, B = cholesteryl TMS ether, C = 24α-ethylcoprostanyl TMS ether, D = β-sitosteryl TMS ether.

Table II shows the relative amounts of fecal sterols. Coprostanol does not appear in feces until the age of 5 to 12 months. Great individual variations are noted. Thus, a few individuals excrete coprostanol at the age of 5 to 6 months, while others do not show any fecal coprostanol even at the age of 1 1/2 years. Furthermore there are considerable variations in successive samples from the same individual. Thus, the subjects 10 and 20 show conversion of cholesterol to coprostanol on one occasion, while no fecal coprostanol could be detected one week later. Finally the degree of conversion shows a tendency to increase with age. All subjects under 1 year of age show less than 34 per cent conversion while those more than one year of age often show more than 60 per cent conversion.

TABLE I Retention times relativ to cholesterol of the fecal sterols and their trimethylsilyl ethers on 1 % SE-30 column

Compound	OH	TMS
Coprostanol	0.87—0.92	0.97—1.03
Cholesterol	1.00	1.26
Campesterol	1.31—1.35	1.62—1.67
24α-Ethylcoprostanol	1.50—1.54	1.62—1.67
β-Sitosterol	1.64—1.72	2.03—2.20

OH = sterols with free hydroxyl group
TMS = trimethylsilyl ethers

The predominant plant sterol found in the feces is β-sitosterol. Campesterol is also found, but this sterol is of a quantitatively minor importance. The appearance in feces of 24α-ethylcoprostanol, the saturated derivative of β-sitosterol, equals in time the appearance of coprostanol. The variations seen in the occurrence of β-sitosterol and campesterol in feces are probably attributable to the differences in diets.

Discussion

The purpose of the present investigation was to study the predominant sterols in feces of infants with special reference to the ratio between cholesterol and coprostanol. It should therefore be stressed that no attempts were made to identify minor components.

The conversion of cholesterol to coprostanol in the human intestinal tract was shown t begin at around one year of age. The occurrence of coprostanol in feces

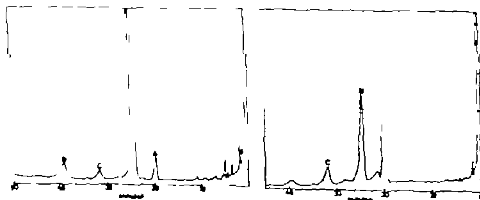


Fig 1 Gas-liquid chromatographic analyses (GC-MS instrument) of the TMS ethers of fecal sterols from subjects 26 (left) and 24 (right)

TABLE II. Ratios between neutral steroids in infant feces

Subject	Age in weeks	Ratios coprostanol : cholesterol campesterol + 24α-ethylcoprostanol β-sitosterol				Per cent conversion of cholesterol to coprostanol
1	4	—	1.00	—	—	—
2	6	—	1.00	—	0.26	—
3	9	—	1.00	—	0.09	—
4	9	—	1.00	—	0.09	—
4	10	—	1.00	0.04	0.15	—
5	9	—	1.00	—	0.07	—
5	10	—	1.00	—	0.06	—
6	12	—	1.00	—	—	—
6	13	—	1.00	—	—	—
7	13	—	1.00	—	0.11	—
7	14	—	1.00	—	0.18	—
8	15	—	1.00	—	—	—
9	18	—	1.00	—	—	—
9	19	—	1.00	—	—	—
10	20	0.22	1.00	—	0.15	18
10	21	—	1.00	—	—	—
11	21	0.08	1.00	—	0.17	7
12	22	—	1.00	—	0.27	—
13	27	—	1.00	—	0.25	—
13	28	—	1.00	0.04	0.20	—
14	27	—	1.00	—	—	—
15	27	—	1.00	—	0.36	—
5	28	0.12	1.00	—	0.29	11
	34	—	1.00	—	0.22	—

The smallest ratio detectable was 0.04

was subject to great variations, both when comparing different individuals and when considering a single individual. The conversion of β-sitosterol to 24α-ethylcoprostanol appeared simultaneously with the saturation of cholesterol to coprostanol.

Coleman and Baumann (1957a) found that rat weanlings excrete coprostanol. This is in contrast to the findings described above for humans, where it takes about one year for the microorganisms responsible for the conversion to become established and active in the intestinal tract. However, these findings are in good agreement with the investigations of Encrantz and Sjövall (1959) where a bile acid-converting microorganism known to be present in adults was found not to be active in infants 1 year of age.

Eneroth *et al.* (1964) investigated the excretion of fecal neutral steroids in normal human adults and found a relatively constant ratio between cholesterol and coprostanol. Thus, the conversion of cholesterol to coprostanol was around 85 per cent.

Subject	Age weeks	Ratios* coprostanol cholesterol campesterol + 24α-ethylcoprostanol β-sitosterol				Per cent conversion of cholesterol to coprostanol
16	33	—	1.00	—	0.18	—
17	35	—	1.00	—	0.27	—
17	36	—	1.00	—	—	—
18	44	—	1.00	—	0.08	—
18	45	—	1.00	—	0.11	—
19	45	0.08	1.00	—	0.19	7
20	49	0.51	1.00	—	0.25	34
20	50	—	1.00	—	0.21	—
21	51	—	1.00	—	0.16	—
21	52	—	1.00	0.05	0.22	—
22	52	—	1.00	0.05	0.15	—
23	60	0.06	1.00	—	—	6
24	64	3.74	1.00	0.81	—	79
24	65	1.46	1.00	—	0.26	59
25	67	3.41	1.00	—	—	77
26	76	0.10	1.00	0.05	0.11	9
27	78	0.29	1.00	0.06	0.11	22
28	80	—	1.00	—	—	—
28	82	—	1.00	—	0.19	—
29	86	1.53	1.00	0.55	0.35	60
29	87	0.41	1.00	—	—	29
30	88	0.06	1.00	—	0.26	6
30	90	0.14	1.00	0.12	0.29	12
31	90	4.90	1.00	1.00	—	83

The figure was quite constant for the same individual on different occasions, and also varied very little between different subjects on different diets. It thus appears that normal adults have a relatively stable cholesterol-converting microflora in clear contrast to infants where great fluctuations in the cholesterol-converting capacity indicate an instability of the corresponding flora.

Coleman and Baumann (1957b) showed that β-sitosterol was converted *in vitro* to 24α-ethylcoprostanol by cultures of intestinal microorganisms which were also capable of saturating cholesterol to coprostanol. Probably the bacteria which perform these reactions are identical. This hypothesis is further supported by the findings presented in the present work.

More work is needed to clarify the different factors that determine the establishment of an active intestinal microflora, capable of converting cholesterol to coprostanol. The data given above do not distinguish between the possibilities that the

appearance of coprostanol in feces may be the result of a recent introduction of active microorganisms into the gut or that it may represent the activation of a previously inactive microflora, perhaps by the establishment of a symbiotic bacterium or by dietary influences. The only way to resolve these problems is to isolate the microorganisms that carry out the reduction of cholesterol to coprostanol.

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Adrenergic Innervation of the Male Reproductive Ducts of Some Mammals III. Distribution of Noradrenaline and Adrenaline

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Abstract

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Distributions of noradrenaline (NA) and adrenaline (A) in testes, epididymides and vasa deferentia were determined by fluorospectrophotometric quantitation [illegible] rats, guinea-pigs, rabbits, and cats. Cat testes and caput epididymides [illegible] centrations than those of other species. Increasing NA concentrations [illegible] epididymides and the largest ones occur in vasa deferentia. Concentrations [illegible] organs examined. The data correlates closely with previous knowledge [illegible] distributions of the adrenergic innervation of these organs.

The present study was undertaken in parallel with a [illegible] localization of noradrenaline (NA) in the adrenergic [illegible] the testis, epididymis, and vas deferens (Norberg [illegible] using the specific fluorescence method of Falck [illegible] et al. 1962. Confirmatory biochemical evidence was [illegible] microscopic observations, and to compare results [illegible] estimates of the acetylcholinesterase (AChE) activity [illegible] a relative estimate of the cholinergic innervation [illegible] of these features might reveal relations not yet [illegible] perplexing problems concerning the innervation [illegible] of these organs. Richardson 196[illegible], 1964 [illegible] adrenaline (A[illegible]) contents of these organs are [illegible] [illegible] result of methods of extraction [illegible] quantitative estimates appear to be unavailable [illegible]

Material and methods

Samples of whole testes (I) caput epididymides (II) cauda epididymides (III) and vasa deferentia (IV) were separated as indicated in Fig 1 and will be designated by the respective Roman numerals in the figures, tables, and text. In testis (I) samples, the tunic is included with the mass of seminiferous tubules and interstitial tissues. Caput epididymides (II) were severed at the testicular junctions with the ductuli efferentes, which were included in this region and from the remainder of the cauda epididymides at the level of the upper corpus. Cauda epididymides (III) included the entire corpora epididymides, and were separated from the vasa deferentia (IV) along the usually well-defined septum that extends caudally from the lower corpus deeply into the cauda. Most of the thick-walled intraepididymal ducts adjacent to the vas deferens proper were included with the latter (IV). These regions were selected upon the basis of prior histochemical and histological observations. Vasa deferentia (IV) were taken in their entirety from their intraepididymal coils (lobe 6C, as designated for rats by Risley and Skrepetos 1964) to their terminations at the urogenital junctions. Dissections of the regions as defined above were made as consistently as possible according to Fig 1

Sprague Dawley rats (n=10) and guinea-pigs (n=5) of approximately similar sizes and ages were killed by concussion and immediate desanguination. Rabbits (n=10) were killed by Lv air or urethane injection, and cats (n=5) were under pentabarbitol anesthesia when organs were removed.

Tissues were minced quickly placed in about 5 volumes of cold 10 % trichloracetic acid, weighed, and frozen for temporary preservation. They were then processed for their NA and A contents by the improved fluorophotometric method described by Euler and Lishajko (1961) This method permits a usual recovery of an approximate 73 per cent of the compounds, and provides data for comparisons of relative rather than absolute, values Organs from both sides of individual rabbits, guinea-pigs, and cats were pooled for each analysis In rats, four organs from two specimens were combined per sample in most cases.

Results

Results for NA and A concentrations and their distributions in rats, guinea-pigs, and cats are summarized in Table I In Table II normal rabbits are compared with observed hypogonadal cases with distinctly lower organ weights. A profile chart (Fig 1) summarizes the NA distributions in the male reproductive ducts of the several species for comparisons of average values for different organs and ranges of variations.

The NA content of the testis (I) is very low for all species except the cat, which has about 10 times the concentrations found in rats and guinea-pigs, and about 5 times that in rabbits. The A content of the testis is also very low in all species, and can be assumed to be negligible (values <0.005 µg/g have not been regarded as valid) These catecholamines evidently are present in the testis in relatively low quantities normally

NA concentrations are higher in the caput epididymides (II) than in the testis (I) by a factor of 3—10 times, but are quite low as compared with the cauda epididymides (III) Highest NA concentrations in region II are in the cat, where about 20 times the average amounts for the other species occurred. This correlates closely with an observed adrenergic innervation of ducts of this region in cats, as compared to its absence except in blood vessel walls in the other species. Concentrations of A are also very low in region II although slightly higher than in the testis.

TABLE I. Noradrenaline and Adrenaline Concentrations in Male Reproductive Organs of Rabbits, Rats, Guinea-pigs, and Cats.

Organ	Number of Samples	Average Single Organ Weights (g) and Ranges	Noradrenaline in μg/g and Ranges	Adrenaline in μg/g and Ranges
A. RABBITS (c. body wts. incomplete)				
I. Testis	10	1.930 (0.540—3.140)	0 04 (0 10—0.11)	<0.05
II. Caput epididymidis	10	0.216 (0.070—0.370)	0.23 (0.09—0.50)	<0.05
III Cauda epididymidis	9	0.486 (0.055—0.960)	3.41 (1 07—7 05)	0.17 (0.00—0.64)
IV Vas deferens	10	0.224 (0.100—0 410)	*6.87 (5 49—9 17)	0.22 (0 07—0.37)
B. RATS (322g. ave. body weight)				
I. Testis	*6	1.326 (0.175—1 445)	0.03 (0.00—0.08)	<0.05
II. Caput epididymidis	5	0.193 (0 185—0.203)	0.08 (0.03—0 17)	<0.05
III Cauda epididymidis	*6	0 160 (0.080—0.205)	0.54 (0.39—0.78)	0.07 (0.00—0 14)
IV Vas deferens	5	0.092 (0.075—0.108)	9 72 (7 79—11 74)	0.05 (0.00—0 15)
C. GUINEA-PIGS (385.5g. ave. body weight)				
I Testis	5	0.660 (0.210—0.920)	0.02 (0.00—0.06)	<0.05
II. Caput epididymidis	5	0.069 (0.035—0.100)	0.25 (0 00—0 71)	0.38 (0.14—0.57)
III Cauda epididymidis	5	0.041 (0.030—0.060)	3.07 (0.88—6.26)	0.37 (0.00—0.49)
IV Vas deferens	5	0.054 (0 050—0.070)	12.78 (9.36—17 60)	0.30 (0.00—0.56)
D. CATS (3,400g ave. body weight)				
I Testis	5	1 431 (0.950—1.805)	0.21 (0.09—0.51)	<0 05
II. Caput epididymidis	5	0.141 (0.065—0.155)	1 14 (0.140—2.39)	<0.05
III. Cauda epididymidis	5	0 154 (0.049—0.170)	1 15 (0 61—2.15)	0.07 (0.00—0 20)
IV Vas deferens	5	0.632 (0 038—0.080)	6.68 (5.22—10 71)	0 12 (0.00—0 64)

Includes two samples each with paired organs from one animal others had four organs from tw animals per sample.

Sjöstrand (1965) reports nearly identical value of 6.768 for five rabbits.

TABLE II Noradrenaline and Adrenaline Concentrations of Normal and Hypogonadal Rabbits
than 0.05 μg/g and values are rounded to the nearest second decimal, except when

Organ	Numbers of Samples	Ave. Wts. in g per Organ	NA μg/g
I Testis			
a. Normal	(7)	2.37 (1.62—3.14)	0.03 (0.01—0.06)
b. Hypogonadal	(3)	0.90 (0.54—1.33)	0.07 (0.02—0.11)
II Caput epididymidis			
a. Normal	(7)	0.25 (0.16—0.37)	0.17 (0.09—0.24)
b. Hypogonadal	(3)	0.137 (0.07—0.15)	0.38 (0.27—0.50)
III Cauda epididymidis			
a. Normal	(6)	0.564 (0.555—0.96)	2.31 (1.07—3.26)
b. Hypogonadal	(3)	0.307 (0.055—0.483)	5.95 (4.12—7.05)
IV Vas deferens			
a. Normal	(7)	0.232 (0.10—0.41)	6.31 (5.49—7.30)
b. Hypogonadal	(3)	0.187 (0.15—0.28)	8.13 (7.18—9.17)

A greater range of variation in NA concentrations appeared in region III than in regions I and II probably because of variations in dissection procedures which could not be as precise as might be desired. Inclusion of portions of region IV with region III would increase the NA content of the latter but inclusion of some of region III with region IV probably would not be recognized in the values. NA concentrations are increased markedly in region III as compared to II or I. The increase was by at least a factor of 10 in the rat, rabbit, and guinea-pig, and corresponds to the observed innervation increase. However in cats, differences did not appear in regions II and III which provides convincing evidence that both the caput and cauda epididymidis of this species have ducts with an adrenergic innervation. Concentrations of A were greater in region III than in the other regions in rats and guinea-pigs, but were greater in the vasa deferentia of cats and rabbits.

The high NA concentrations in region IV are within the ranges of those observed by Sjöstrand (1965) for vasa deferentia of these species. The average value for guinea pigs is somewhat higher than Sjöstrand's figure, but this result can be

Male Reproductive Organs. Ranges of variation are omitted when maximal A values are less
calculated/organ.

NA μg/organ	A μg/g	A μg/organ
0.039 (0.026—0.075)	<0.05	<0.05
0.107 (0.087—0.118)	<0.05	<0.05
0.035 (0.017—0.088)	<0.05	<0.05
0.056 (0.026—0.075)	<0.05	<0.05
1.053 (0.550—1.690)	0.10 (0.00—0.12)	<0.05 (0.00—0.12)
1.714 (0.388—3.105)	0.51 (0.06—0.64)	0.125 (0.00—0.26)
1.430 (0.74—2.30)	0.24 (0.13—0.37)	0.052 (0.022—0.106)
1.563 (1.05—2.563)	0.19 (0.07—0.30)	<0.05 (0.011—0.085)

explained on the basis of an immaturity of our guinea pigs (70—75 days of age). Very few sperm had reached the vasa deferentia in our animals, although a few were observed in smears from the testis and epididymis. Higher NA concentrations characterize juvenile or hypogonadal individuals because of reduced organ weights due to the degree of development of tissues other than those containing NA. Similar results were found in several individual rabbits, as summarized in Table II.

Discussion

The only previous measurement of testis NA concentration was made by Euler (1956) who reported an amount of 0.04 μg/g for the bull testis. This approximates the quantity found in the rabbit testis. The highest NA content for testes was in the cat. This observation agreed with the histochemical findings that fluorescent adrenergic terminals were associated with some blood vessels that penetrated more deeply into the testis interior than in the other species. Van Campenhout (1949) reported

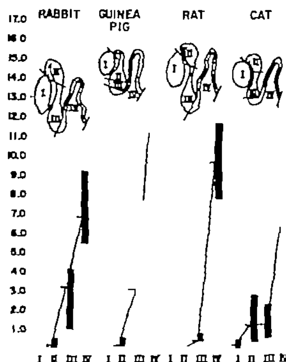


Fig. 1. Graphic summary of the distributions and concentrations of noradrenaline in the male reproductive ducts of the rabbit, rat, guinea-pig, and cat. The diagrams in each column depict the approximate points of separation of regions I, II, III, and IV for analysis. Mean values and ranges of variation of the NA content in µg/g are given for each region as indicated by the points and bars respectively. Roman numerals refer to organ regions in upper figures; Arabic numerals to µg/g NA.

a strong innervation of boar and cat testis interstitial cells, bud did not find the same kind of autonomic innervation in the rat testis after silver staining. The fluorescence method for NA in nerves of the cat testis did not demonstrate a plexus similar to the one he described in detail for the boar testis, however. The NA quantity in the cat testis does not appear to be great enough to confirm his interpretation, but the low NA of the rat testis is consistent with his observations. Such 1963) also reports that interstitial cells of the dog, guinea-pig and rat do not have an innervation following Maillet's osmic acid-zinc iodide method. Risley and Skrepetos (1964a) did not find an innervation of internal testicular structures in rats or guinea pigs following AChE localization methods. From the known histochemical facts concerning testicular autonomic innervation, peripheral autonomic nerves are associated with mostly peripheral blood vessel walls and only to a limited degree in deeper tissues. Therefore, regulation of blood flow seems to be a major function of the limited adrenergic innervation in this organ.

Baumgarten and Holstein (1967) observed fluorescent adrenergic nerve fibers in the intertubular tissues of the human testis, where they were mainly associated with blood vessel walls. The proximity of interstitial cells to the varicosities of the occasional peripheral fibers of the blood vessel walls and the blood vessels, themselves, indicated to them that some interstitial cells possibly were innervated. Norberg et al. (1967a) observed conditions resembling those figured by Baumgarten and Holstein (1967) but were not fully convinced that the interstitial cells were more than re-

cidentally or occasionally innervated in cat testes. Occasional fibres also were observed marginally along some seminiferous tubules, but these were so rare that it was thought that these were blood-vessel components mainly which could play only a relatively limited role in seminiferous tubule function, if any

Falck, Owman and Sjostrand (1965) observed the presence of adrenergic terminals in the cauda epididymidis of the guinea-pig, but no quantitative data on the NA or A content of the epididymis are available. In the ductuli efferentes and caput epididymidis (II) of the cat, the NA content exceeded that in th other species, which agrees with the observation that these duct walls are innervated (Norberg *et al.* 1967a) Fluorescent adrenergic nerves were observable only in blood vessel walls in the other species. This indicates that species differences exist and that the facts must be ascertained for each species.

In the cauda epididymidis (III) the NA concentrations are much greater than in regions I and II of the rat, rabbit, and guinea-pig In the cat, however regions II and III are quite similar to each other indicating again that the histochemical observations are consistent with the quantitative estimates. Risley *et al.* (1964) found a similar difference in the low quantity of estimated AChE activity in region II as compared with region III of the rat epididymis. This correlated with an observed absence of AChE reactive nerves in region II as compared to region III (Risley and Skrepetos 1964) The quantitati e data for NA content and AChE activity do not provide a basis for distinguishing between cholinergic and adrenergic innervations of region III but do demonstrate a comparative relative similarity in the distributions of both substances in regions II and III

Lower NA quantities appearing in region III of the rat could be due to several factors. In dissection procedures, less of region IV might have been included in the samples than in the other species, for example. On the other hand, histochemical observation (Norberg *et al.* 1967) demonstrated that an adrenergic innervation of the laminae propriate of the duct walls in regions III nd IV was not present in the rat, but did occur in the other species. Lower NA quantities therefore would be expected. Koelle and Jacobow tz (1965) however did not find adrenergic terminals in the lamina propria of the vas deferens of the guinea-pig rabbit, and cat, as Norberg *et al* (1966) did. The smooth muscle walls of the cauda epididymidis of rats also may be more weakly innervated than n the other species.

The comparative data of Sjöstrand (1965) on the NA content of vasa deferentia of numerous mammals are confirmed by our data for the species examined. The high NA quantities for this organ correlate well with th extensi e adrenergic innervation of the muscular wall (Falck 196_, Owman and Sjostrand 1965) as demonstrated histochemically A high AChE activity has also been reported for this organ in the rat (Risley t al. 1964 Ohlin and Strömblad 1963) but the data do not supply discriminating evidence concerning independent distributions of cholinergic and adrenergic innervation (Jacobow tz and Koelle 1965) H pogastric denervation does not reduce the NA values (Sjöstrand 1962, 1965 or th fluorescence of the adrenergic terminals of this organ (Jacobowitz and Koell 1965 However Nor

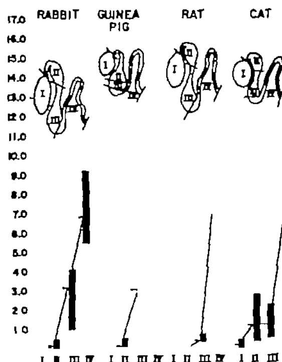


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centrations in the cauda epididymidis. Quantitative values correlate closely with and corroborate previous histochemical descriptions of adrenergic nerve distributions.

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berg *et al* (1967b) vasectomized rats and cats and terminals disappeared after several days on t site. They remained present at the prostatic e between the hypogastric nerve and the terminal *et al.* 1963)

Higher NA concentrations appeared in organ *and in 3 hypogonadal rabbits. An extreme NA con* in the vas deferens of the smallest guinea pig, whic exception is omitted from the group tested and only average NA concentration would be 11 6 ng/g with a with value given in Table I C IV) Sjöstrand (1965) tion of 10 0 ng g for the guinea pig vas deferens. It is p reported for normal rabbits in Table II represent truer v the total number of the rabbits examined (Table I) T showed greater a erage NA concentrations in all regions. I butions in the several regions showed the typical progressive i with the distribution of NA-containing nerve tissues.

Values obtained for the concentrations of A are uniformly lo organs examined. The guinea-pig tissues contained more A than t this may be due to the immaturity of the animals used as the s in hypogonadal rabbits. Sjostrand (1965) reports a mean value of the as deferens of the macaque, and found it to be present in semin prostate glands of several other species. The presence of A in signific is regarded by Euler (1956 1961) to indicate the presence of chromaff Euler (1934 and Sjostrand (1965) demonstrated their occurrence in c prostat gland respectively From the low A concentrations and appare of identified chromaffin cells in the present material adrenaline evident produced in ignificant quantities. The cauda epididymidis and vas defer tained the largest quantities however but testis tissues were so lacking in the measurement were below limits of accurate determination. It is conc theref re that techolamines cannot be regarded as important normal constit of the test and caput epididymis in the rat, rabbit, and guinea-pig. Difference the A conc nt ations in the several regions is not great enough to be of importa although it ppears as a c nst t ent of seminal fluids (Mann 1964)

Summary

Distributions of nor drenaline and adrenaline quantities in male reproductive organs of rats guinea-pigs bbits, and cats were estimated fluorophotometrically Both compounds occur in ery l w amounts in the testis, and only slightly higher alues appear in the caput epidid midis The cat has the greatest NA concentrations in these two organs, which orrelates with the occurrence of a greater adrenergic innervation. Increased alues in the cauda epididymidis and vas deferens correspond with an increasing inner tion in other species. Adrenaline is present in greater con-

hematuria. 2 or 3 urine collecting periods were made per experiment (*cf* Table I and II) *Dur g th rin collection blood was nt neously and simultaneously sampled f om a super ficial enal ein d p enal vein and f om femo al artery at the sam pe d* according to the technique described (Nissen 1965 and 1966) Tw or three blood sampling periods wer made per rine collecting period.

Priming doses and sustaining doses of inulin and p-aminohippurate (PAH) dissol ed in 0.9 per cent NaCl were administered in a jugular ein. The sustaining solution was given t rate of some 0.4 ml/min.

The blood pressure was measured by means of mercury manometer connected to carotid artery

Experiments ith fluid load g The first urine collecting period, in hich only the inulin-PAH solution was given, served as control period for later periods. I these an osmotic diuresis was produced in 3 expts. by continuous i infusion of 20 per cent *mannitol* in 0.9 per ent NaCl at rates arying between 1.2 and 2.8 ml/min the infusion was preceded by priming dose (8 to 90 ml of the same solution)

Saline diuresis was produced in 5 expts. by i administration of *Ri ger' sol tio* (NaCl 145 meq/l, KCl 3.4 meq/l, $CaCl_2$ 2.5 meq/l, N HCO_3 2.4 meq/l, $MgCl_2$ 2.0 meq/l osmolality measured by the method of freezing point depression 300 mosm/kg) 100 to 700 ml wer given in 10 to 20 min. Following this constant infusion of the same solution was given t rate of 2—3.5 ml/min. In one of the Ringer experiments *plasma* taken from another cat immediately before the experiment, was also administered. I another experiment plasma alone was given. *D ervation* as carried out in few instances by cutting all visible nerv fibers along the great renal essels and applying cotton pads moistened with 2 per cent xylocain in the hilus region.

Cont ol periments were performed in order to ascertain that the ratio between the deep and superficial filtration fraction was reasonably onstan throughout 2 or 3 urine collecting periods if mannitol solution, Ringer fluid or plasma ere not given. (After the last period Ringer fluid or plasma as at times administered and an additional period of blood and urine sampling then made)

A minimum of 25 min elapsed both in the fluid loading and in control experiments before new urine collecting period was started

The plasma was analyzed for protein, inulin and PAH and the urine for inulin and PAH. All light absorbances were read on Zeiss spectrophotometer The *prote* was determined in duplicate by biuret reaction (Gornall, Bardawill and David 1949) 100 µl of plasma were used per protein determination. A ery slight onvexity of the standard curv against the absorbance axis was taken into consideration by adding 0.010 or 0.015 absorbance units to the blank alue before subtraction from the sample alue Actuall this small correction was immaterial for the present purpose since onl fractional (enous/arterial) oncentrations occu in the formulae used

I li as analyzed according to Bojesen (1952 Duplicate protein free filtrates were made using 100 µl plasma or diluted rine per deproteinization T determinations were made on each deproteinized sample The inulin determinations er corrected for the presenc of glucose hich produced an absorbance of 1 per cen of that of inulin equal concentr tions, (*cf* Nissen 1966)

4 analyses for *PAH* (Smith *et al* 1945 were also made on each plasma or urine sample 300 µl ere used per deproteinization The olor developed hen analyzing superficial renal enous plasma at the low arterial plasma concentrations was only some 0.01 absorbance units abov the blank alue (some 0.006) determined in arterial plasma draw before the PAH administration A the magnitude of the blank absorbance as critical est ere made in one experiment to ascertain that he blank alues of enou plasma from he two drainage areas actually equalled that of arterial plasma

The *osmolality* of the urine samples as measured by he meth d of freezing poin depression (Advanced Instruments, Newton H ghl nd Mass af er ppropriate dilution of the urines th ter

The *filt ation fractio* of plasma er the superficial nd deep region of the orte as calculated by means of formula previousl derived Nisse 1966

$$FF \quad \frac{1}{I} \quad \frac{N \quad A \qquad N \quad N}{N \quad A}$$

 here N_P A_P N_I and A_I ar plasma oncen protein P and inu I d nase blood N and arterial blood A When l i nase blood is from l paula renal eins the formula yields filtration frac entativ for abou oute half of the cortex. Filtration fractions for the inner ortex are obtaine the calculations re based on oncentrations in blood from le nal veins. fig =1 is factor corre

TABLE I. Mannitol and Ringer loading

Exp. no.	Event	Number of Periods*	FF_s fw*	FF_D fw	FF_D/FF_s
1	no load	3	.341	.321	.94
	mannitol	3	.290	.239	.82
2	no load	5	.266	.208	78
	mannitol	5	094	051	.54
3	no load	5	.368	.269	73
	mannitol	5	.211	119	.56
4	no load	2	.243	.245	1.01
	115 ml Ringer	2	.270	.217	.80
	+200 ml Ringer	2	.201	148	74
5	no load	2	.335	.318	.95
	110 ml Ringer	2	.337	.259	77
	+170 ml Ringer	2	.300	198	.66
6	no load	2	.278	.252	.91
	110 ml Plasma	2	.201	157	78
	+115 ml Ringer	2	160	122	76
7	no load	2	.354	.313	.88
	Denerv	2	.338	.247	73
	110 ml Ringer	2	.236	134	.57
8	no load	2	.266	.299	1 12
	Denerv	2	307	.317	1 03
	115 ml Ringer	2	.271	.213	79
	no load	2	.310	.232	75
	Denerv	2	.293	.233	79
	100 ml Plasma	2	177	137	.77

* Number of blood sampling periods per urine collection period. The individual FF_D and FF_s values, respectively scattered very little within each urine collecting period, therefore FF and FF values times fw) given are averages.

$FF_{D\ or\ s}$ times fw (fw = about 1 05) is given instead of $FF_{D\ or\ s}$ because the absolute magnitude of the filtration fractions is of minor importance, and because fw cancels in the ratio FF_D/FF_s (cf. methods

the concentration of albumin in plasma to the concentration in the ultrafiltrate. It cancels out when, as in the present study, merely the ratio between the deep and superficial filtration fraction is considered

The *total renal plasma flow*, RPF_{el}, was calculated by dividing the urinary excretion rate for PAH by the average of the arterio-venous concentration differences in the superficial and deep drainage areas. Actually when using the average one presupposes the two venous drainage flows to be of equal magnitude this is only approximately true

Results

The results of fluid loading and control experiments are shown in Tables I and II respectively. The majority of the FF_D/FF_s ratios were below unity thus confirming

$RPF_{tot.}$ (ml/min)	Cl_f (ml/min)	D (ml/min)	Osm. (mosm/kg)	BP (mm Hg)
25	—	025	—	130
26	—	1 123	—	170
—	9 1	100	—	135
—	12.7	413	—	135
14	3.9	.035	—	150
43	6.2	2.03	—	140
33	8.4	.027	—	135
52	12.3	168	—	130
57	9.2	.388	—	125
27	8 6	.040	—	135
39	10.4	.261	—	145
49	11 1	.558	—	140
32	7.8	.079	—	125
52	9 0	141	—	140
62	9 4	.309	—	140
31	10 1	.035	2 350	140
36	10.0	.087	1 305	140
53	9.0	.354	623	135
44	12.1	.031	2 010	140
39	11.6	.055	1 685	140
42	11.0	178	882	150
41	10.3	.073	1 050	120
43	11.0	094	1 000	120
69	10 8	.374	496	150

the earlier observations (Nissen 1966) Both FF_D nd FF_a decreased when *man tol* ol tion was given (exp. 1 and 3) but the former to a greater degree than the latter as indicated by a fall in the FF_D FF_a ratios. The a erage ratio between FF_D FF measured during mannitol loading nd FF_D FF measured before loading was *0.79* *Ringer* infusion also lowered the FF_D FF ratios both in the innervated and the denervated kidney th average ratio between FF_1 FF measured after Ringer loading nd before the loading was *0 77* With lower Ringer loadings (bout 100 ml) the filtration fraction of the plasma entering the inner half f th rtex (FF_D) decreased that of the plasma ntering th outer cortex (FF) remained n-changed (exp. 5 T ble I nd VII Table II ncreased slightly (exp. 4 and V) or decreased (in the denervated kidneys, exp 7 and 8) W th higher R nger loadings (about 100+150 ml) or f plasma w previously given, both FF nd FF_f de-creased (exp. 4 5 and 6)

In the control experiments the erage ratio between FF FF measured in the

TABLE II. Control experiments

Exp. no.	Event	Number of Periods	FF_s f_w	FF_D f_w	FF_D/FF_s
I	no load	3	.318	.293	.92
	no load	3	.388	.350	.90
II	no load	3	.294	.278	.93
	no load	3	.306	.281	.9
III	no load	3	.336	.274	.81
	no load	3	.285	.220	.77
IV	no load	2	.300	.235	.78
	no load	2	.309	.250	.81
V	no load	2	.342	.335	.98
	no load	2	.35_	.352	1.00
	no load	2	.342	.310	.91
	100 ml Ringer	1	.364	.267	.73
VI	no load	2	.230	.232	1.01
	no load	2	.274	.276	1.01
	no load		.225	.227	1.01
	100 ml Plasma	1	190	175	.9_
VII	no load	2	.343	.289	.84
	no load	2	.332	.267	.80
	no load	2	.308	.256	.83
	110 ml Ringer	1	.305	.235	.77
VIII	no load	2	.390	.403	1.03
	no load	2	.339	.313	.92
	no load	2	.351	.326	.93
	70 ml Plasma	1	.284	.273	.96

second or third urine collecting period, and FF_t/FF measured in the first urine collecting period was 0.97. Thus no significant fall occurred in the control experiments. The ratios 0.79 and 0.77 (see above) differed significantly from 0.97 ($p <$ 0.001).

Plasma infusion produced a fall in the FF_t/FF ratios in 2 out of 4 expts. (exp. 6 and VI). The average ratio was not significantly changed compared to the control experiments ($p >$ 0.0). *Denervation* caused a fall in 2 out of 3 expts. (exp. 7 and 8).

The inulin clearance (Cl_i) and the total renal plasma flow (RPF_{tot}) often increased during fluid loads. The urine flow (D) always increased. The blood pressure (BP) remained unchanged with Ringer loading but increased somewhat upon plasma infusion (Table I and II). The increase in diureses provoked by fluid loading or denervation was associated with a fall in the urine osmolality (Osm). In all the experiments where osmolality measurements were made

RPF (ml/min)	C_{I} (ml/min)	D (ml/mm)	Osm. (mosm/kg)	BP (mm Hg)
—	9.2	114	—	140
—	7.0	.061	—	120
—	8.9	.091	—	155
—	8 8	.089	—	165
—	8.2	143	—	170
—	6 4	168	—	180
29	7.6	.027	—	120
22	5.8	.020	—	130
32	9 6	025	2 350	145
27	8.8	.023	2 360	145
26	8.5	.022	2 270	135
31	9.2	111	1 110	150
22	5.0	.033	1 010	110
25	5.6	.042	1 110	120
34	6 7	.048	1 135	115
48	8.1	148	727	140
34	10 6	.034	2 100	125
42	11.3	.051	1 665	130
32	8.4	.036	1 460	130
38	10.4	143	822	135
23	8.2	018	2 420	120
28	7 7	023	2 070	115
23	6.8	019	2 150	120
32	7 1	020	2 190	120

Discussion

The efferent arterioles of the j tamedullary glomeruli supply the med lla. In these glomeruli Zlabek (1957 and b described relatively thick capillary short-circuits connecting the afferent w th the eff rent arterioles, causing the blood to partly bypass the sites of ultraf ltration. Granberg Lagergren and Ljungqvist (1964) suggested that the filtration fraction in the juxtamedullary glomeruli was low d to the short-circuit, and Nissen (1966) explained the finding of a comparativel low filtration fraction in the deep drainage area (FF_D) as a reflection of the fact that the juxtamedullary glomeruli constitut part of the glomeruli contained n th deep cortex. Tru extraglomerular essels (arteriolae rectae vera Isaacs-Ludwig arterioles, and spiral arteries) can also be responsible for a diversion of blood f the sit of ltrafiltration, however to lesser extent since they are rare

The fall in the FF_I at rather fixed FF_R values, observed with smaller Ringer infusions in the present experiments, can most naturally be accounted for by a preferential rise in the medullary plasma flow viz. such a rise will result in FF_D being governed to a greater extent by the presumably low filtration fraction of the plasma traversing the juxtamedullary glomeruli (plus extraglomerular vessels). With larger fluid loadings FF_R also decreased, but to a lesser extent than FF_D as indicated by a decrease in the ratio FF_D/FF_R.—Other interpretations are possible e.g. that the plasma flows entering the different glomeruli are uniformly elevated while the filtration rates increase proportionately less in the inner glomeruli than in the outer glomeruli. However if this was the main reason for the decrease in FF_D/FF_R the decrease should always be accompanied by a conspicuous rise in Cl_I and a fairly large rise in RPF_{tot} such rises were not always seen (exp. 7, 8, V). Also the finding of a relatively large decrease in the extraction fraction of PAH in the deep drainage area at fluid loading seems to harmonize better with the idea of a preferential increase in the blood flow through the juxtamedullary glomeruli (cf. Nissen 1968).

In any case the results show that plasma is handled differently by glomeruli of the outer and inner cortex. Therefore, in the following some peculiarities in the morphology of glomeruli with afferent and efferent arterioles will be referred to with the intention of showing that, in fact there is also a structural basis for supposing that the flow through the corpuscles is non-uniformly regulated throughout the cortex. Edwards (1956 man) found larger luminal and total diameter in the juxtamedullary efferent arterioles than in the efferent arterioles of the cortical glomeruli, and due to the relative thickness of their walls (15 µm in the juxtamedullary efferents compared with 4 µm in the cortical efferents) the author concluded that they contained large amounts of smooth muscle. Similar conclusions were drawn by Smith (1956 man) Moffat and Fourman (1963) and Fourman and Moffat (1964 (? and other mammals) observed filling defects in the efferent arterioles of the juxtamedullary glomeruli in specimens injected post mortem with indian ink and Neoprene latex. The defects, which were always in the same position, were considered the results of a sphincteric action of the proximal part of the vessels. They were often sufficiently well marked to cut off the arterioles from the vasae rectae so that the arterioles appeared to end in the subcortical capillary plexus, while the vasae rectae appeared to begin in this plexus.

The deep (juxtamedullary) corpuscular units have a low content of renin in the juxtaglomerular apparatus (Peart, Gordon, Cook and Pickering 1956, Bing and Wiberg 1958, Brown et al. 1965). The number of epitheliod cells and macula densa cells is small, and the granulation poor; in contrast Goormaghtigh cells occur frequently in deep nephrons (Fårup 1965).

The findings of Fourman and Moffat (1964) and Edwards (1956) indicate a particularly active regulation of the medullary blood flow. The functional significance of the characteristics of the deep juxtaglomerular apparatus cannot be properly evaluated because understanding of the role of the renin-angiotension system in the regulation of the glomerular blood flow and the glomerular filtration rate is still

hypothetical and controversial (Leyssac 1966, Thurau 1966) however it seems probable that the different structure and renin content of the deep juxtaglomerular apparatus are intimately associated with special characteristics of the blood flow through the deep glomeruli.

Barajas and Latta (1963) and Fårup (1965) reconstructed from serial sections the course of the distal tubule with respect to the arterioles of both superficial and deep glomeruli. While the former authors observed an obligatory location of the macula densa at the efferent arteriole, Fårup (1965) found the location variable contact with the afferent vessel being slightly more common than with the efferent vessel. There was no systematic difference between superficial and deep glomeruli in this respect. It is well established that juxtamedullary glomeruli sometimes have two efferent arterioles (for references, see Fourman and Moffat 1964) a wide one supplying the medulla and a smaller ending in the deep cortex. As precise knowledge of the contact between a distal tubule and the afferent and efferent arterioles may be decisive for the understanding of the function of the juxtaglomerular apparatus, further studies on the anatomy of juxtamedullary and cortical corpuscles are needed —e.g. does the distal tubule of glomeruli with two efferent vessels contact the wide medullary or the thinner cortical vessel or both.

It is reasonable to suggest that the large decrease in FF_D compared to that of FF_S is due to a more or less isolated rise in the flow through those glomeruli possessing short-circuits, and that the rise is caused by a dilatation of the thick muscular efferent arterioles of the same glomeruli. The underlying mechanism for this reaction is unknown. Humoral agents may play a role since the reaction can be produced in the denervated kidney and administration of fresh donor plasma yields variable results. The renal nerves may also be involved however there is insufficient information to determine their significance.

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The Extraction Fraction of p-Aminohippurate in the Superficial and Deep Venous Drainage Area of the Cat Kidney

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Abstract

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At plasma concentrations below 40 µg/ml the average extraction fraction of PAH was 0.95 in the superficial drainage area (consisting of approximately the outer half of the cortex) and 0.82 in the deep drainage area (the inner cortex + the medulla). Loading with Ringer's fluid, mannitol solution or plasma generally resulted in a decrease in the extraction fraction of both areas. The two extraction fractions fell to 0.86 and 0.64 respectively. The incompleteness of the extraction cannot be attributed to admixture of blood from the fibrous and fatty capsules of the kidney or to impurities in the PAH preparations. The erythrocytes showed a very low permeability to PAH; correction of renal venous plasma concentrations for diffusion of PAH from erythrocytes to plasma is unnecessary in the cat.—The Reubi principle of calculating the medullary plasma flow as the difference between the total renal plasma flow and the clearance of PAH is misleading in the cat, as it presumes complete extraction in cortical tissue.

The renal excretion of p-aminohippurate (PAH) occurs by the combined processes of glomerular *ultrafiltration* and tubular secretion. In the *mammalian* kidney the rate of excretion divided by the rate of entrance via the renal arterial system (the extraction fraction) generally approaches values between 0.75 and 0.95 with decreasing plasma concentrations.

Nissen (1965) observed that the extraction fractions of PAH in the superficial and deep venous drainage areas of the cat kidney averaged 0.94 and 0.82, respectively, at low plasma concentrations. The superficial area consists roughly of the outer half of the cortex and the deep area of the remaining cortex plus the medulla. The relatively low extraction fraction of plasma traversing the deep area indicates that medullary tissue is responsible for the incomplete extraction of PAH in the kidney to a comparatively large extent; however, the fact that the extraction fraction in the superficial drainage area is also below unity shows that neither is cortical tissue capable of completely clearing the plasma. The results of additional experiments dealing with the renal extraction of PAH in the non-hydrated and hydrated cat are described in this paper.

The volumes of plasma which enter and leave a renal venous drainage area per time unit are denoted ARPF and VRPF respectively. A_{PAH} is the plasma concentration of PAH in arterial blood. V_{PAH} *is the plasma concentration in venous blood from the area in question.* The net rate at which PAH is eliminated from the area along the tubules ($Elim_{PAH}$) equals the difference between the rates at which PAH enters and leaves the area along the blood vessels, the loss in the lymph vessels being negligible as compared to the tubular elimination rate

$$Elim_{PAH} = ARPF \cdot A_{PAH} - VRPF \cdot V_{PAH}$$

$$\text{or } \frac{Elim_{PAH}}{ARPF \cdot A_{PAH}} = \frac{ARPF/VRPF - V_{PAH}/A_{PAH}}{ARPF/VRPF} \qquad (1)$$

$Elim_{PAH}/(ARPF \cdot A_{PAH})$ defines the extraction fraction of PAH in the area. It indicates the rate of extraction (or elimination) divided by the rate at which PAH is transported to the area by the arterial plasma.

As the glomerular capillaries are impermeable to protein, and as the amount of protein leaving the area via the lymph may be regarded as negligible we get

$$ARPF \cdot A_P = VRPF \cdot V_P$$

$$\text{or } \frac{ARPF}{VRPF} = \frac{V_P}{A_P} \qquad (2)$$

where A_P and V_P are the plasma concentrations of protein.

Insertion of (2) in (1) yields an *extraction fraction* of

$$\frac{Elim_{PAH}}{ARPF \cdot A_{PAH}} = \frac{V_P/A_P - V_{PAH}/A_{PAH}}{V_P/A_P} \qquad (3)$$

Since V_P/A_P does not differ more than 0.1 from 1.0 and V_{PAH}/A_{PAH} seldom exceeds 0.30 the extraction fraction may be approximated to $1 - V_{PAH}/A_{PAH}$. The latter term was used by Nissen (1965); in this report the extraction fraction is calculated throughout according to equation 3.

Equation 3 presumes that none of the PAH contained in the erythrocytes of the inflowing plasma diffuses into the drainage plasma as the result of the steep fall in the plasma concentration created by the active tubular transport. The permeability to PAH of cat erythrocytes was therefore investigated.

Methods

The results were obtained in 23 expts. described by Nissen (1966 and 1968); only 3 expts. in which occlusion of the ureter were performed, and the permeability experiments (see below) are new. Blood was drawn continuously and simultaneously from a vein draining the superficial part of the kidney, from another vein draining the deep part of the kidney, and from a femoral artery (Nissen 1966). The PAH (quality "pure") was supplied by two manufactories, Koch-Light Laboratories, England and Fluka, Switzerland. In 10 of the experiments Fluka preparation was used.

Mannitol and *Ringer* solution as well as inulin and PAH solutions were administered as described (Nissen 1968). *Occlusion of the ureter* was carried out in 3 experiments after loading with a few ml of 6 % NaCl and 100 ml 0.9 % NaCl. The occlusion was maintained for 44, 63 and 53 min, respectively.

The permeability of the erythrocyte membrane to PAH was investigated as follows:

1. In 5 expts. in which fairly constant arterial plasma PAH concentrations were sustained, the red cell concentration of PAH was determined in the following manner: 10 ml of heparinized blood were centrifuged at 3 000—4 000 r.p.m. for 30 min and the supernatant plasma carefully removed by a capillary pipette. About 1 volume of distilled water was stirred into the cells to permit decantation, and the mixture was transferred with minimal washing into a graduated cylinder and the volume made up to 2.5 times the volume of cells taken, as determined by the weight of the red cells divided by their specific gravity (1.098 g/ml). The mixture was then stirred to facilitate hemolysis. 500 µl of the *hemolyzed suspension* were treated in the same manner as the plasma samples. A blank erythrocyte "concentration" determined from blood drawn before the PAH infusion was subtracted from the concentration determined from the PAH-containing cells. The recovery of PAH (in the hemolysate) was found in one experiment to be 87 per cent, a correction for this was applied. Inulin does not penetrate the red cell membrane, therefore the inulin concentration in the supernatant plasma and in the hemolyzed suspension allowed us to calculate the plasma volume trapped between the centrifuged red cells. On the basis of this volume the PAH contained in the trapped plasma was corrected for.

2. 1. 3 wer expts. PAH was added to 50 ml blood. The plasma concentrations were followed for 2, 2 and 8 hrs, respectively, samples being taken at intervals increasing from 20 to 90 min in each. The blood was kept in water bath at 35—37 C and gently mixed at short intervals in order to keep the red cells in suspension. T 1824 (Evans blue) had been added to the blood together with the PAH. T 1824 does not penetrate into the erythrocytes and relating the plasma concentrations of PAH to those of the dye (measured spectrophotometrically at 620 nm in plasma diluted 5 times) allows for correction of small changes in the plasma PAH concentrations caused by swelling or shrinkage of the erythrocytes. PAH and T 1824 were also added, in each experiment, to plasma volume (and water volume) in the same mutual proportions as they were added to the blood. The fact that the ratio between the PAH and T 1824 concentrations of plasma was equal in the first sample drawn from the blood volume and from the plasma volume, showed that PAH had not penetrated into the erythrocytes during the short time from addition of PAH till centrifugation of the first blood samples.

The methods for the measurement of the PAH and inulin concentrations and for calculating the total renal plasma flow (RPF $_{tot}$) are described in Nissen (1968).

Results

The extraction fractions of PAH as related to the arterial plasma concentrations of PAH in non-hydrated animals are depicted in Fig. 1 left and right. The extraction fraction of the plasma entering the superficial area was always closer to 1.00 than that of the plasma entering the deep area in the same experimental period. Below 40 μg/ml the fractions reached maximum, the average value for the superficial area being 0.949 (*the distribution was somewhat skewed, only 4 values out of 63 exceeded* 0.970 *the highest value was 0.981*) and for the deep area 0.813 SD = 0.070 n = 63. Above 40 μg/ml the extraction fractions in both areas declined, in fact they converged towards the filtration fractions as one would expect if the secreted amounts

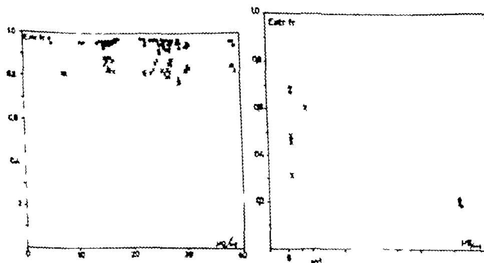


Fig. 1. The variation of the superficial ( ) and the deep (×) extraction fraction of PAH with the plasma concentration of PAH. Abscissa: plasma concentrations. Ordinate: extraction fractions. Results from 23 expts.
Left: concentrations from 0 to 40 μg/ml.
Right: logarithmic concentrations from 40 to 2 600 μg/ml.

TABLE I. The changes in the superficial and the deep extraction fraction of PAH due to fluid loading

Exp no	Event	Number of periods	A_{PAH} (µg/ml)	Superficial area V_{PAH}/A_{PAH}	Superficial area Extr F	Deep area V_{PAH}/A_{PAH}	Deep area Extr F	RPF$_{PAH}$ A_{PAH} (µg/min)
1	no load	3	16	.04	.96	15	.84	404
	mannitol	3	16	.04	.96	16	.83	402
2	no load	3	9	03	.97	.20	.80	331
	mannitol	3	21	.53	.51	78	.24	820
3	no load	3	29	.05	.96	.23	7	411
	mannitol	3	23	.26	77	.59	41	993
4	no load		12	.03	.95	17	.82	411
	115 ml R.L.	2	11	.04	.96	17	.81	520
	−200 ml R.L.	2	9	07	.93	.26	72	310
5	no load	2	23	.05	.95	18	.82	617
	110 ml R.L.	2	20	07	.94	.25	74	768
	−170 ml R.L.	2	17	12	.89	37	.62	830
6	no load	2	23	03	97	17	82	712
	110 ml Pl.	2	10	06	.93	.24	74	339
	−115 ml R.L.	2	9	08	.92	.26	72	339
	no load	2	25	.04	.96	13	.86	786
	Denerv	2	18	05	96	18	.80	646
	110 ml R.L.	2	15	13	.89	36	.61	776
8	no load	2	16	0?	95	16	.83	685
	Denerv		16	06	.94	16	.83	640
	11 ml R.L.	2	15	11	.89	.28	70	656
9	no load	2	2?	10	.91	.34	.63	1 055
	Denerv		24	10	91	.32	67	1 080
	100 ml Pl	2	20	28	73	.52	47	1 385

The individual parameters scattered only little within each pair or triplet of sampling periods, therefore   erages of the pairs and triplets are presented.

become small compared with the ultrafiltered amounts, and the fraction of free (non protein bound) PAH in plasma approaches one.

Loading with *R   e   fl id* or *plasma* generally depressed the superficial as well as the deep extraction fraction (exp. 4—9, Table I). The latter decreased more than the former. In some experiments the infusion rate of PAH was set at a lower level during and after the fluid loading thus securing a lower renal PAH load (RPF$_{PAH}$ A_{PAH}) in the experimental periods than in the control periods (exp. 6, 8). The extraction fractions also declined in these instances. The extraction fractions also decreased in the denervated kidney upon fluid loading (exp. 7, 8, 9). The

TABLE II The changes in the superficial and deep extraction fraction of PAH due to ureterocclusion

				Superficial Area		Deep Area		
Exp no.	E cnt	Period-number	Ap n (μg/ml)	V_{PAH}/ A_{PAH}	Extr Fr	V_{PAH}/ A_{PAH}	Extr Fr	RPF, A AH (μg/min)
I	no occl.	1	34	10	.91	.30	69	1 060
	occl.	2	33	16	.83	.37	.62	—
	occl.	3	29	.24	77	49	.50	—
	occl.	4	27	.23	77	.53	47	—
	occl.	5	26	.21	.80	47	.33	—
II	no occl.	1	39	12	89	.38	.61	1 340
	occl.	2	39	17	.84	45	.52	—
	occl.	3	40	.32	70	65	.31	—
	occl.	4	43	41	.60	78	19	—
	occl.	5	42	48	.53	78	.20	—
	occl.	6	4	.57	44	.87	11	—
III	no occl.	1	36	.34	.68	.59	40	1 550
	occl.		38	.52	49	.67	.33	—
	occl.	3	45	66	.34	73	.24	—
	occl.	4	49	7?	.28	79	19	—
	occl.	5	52	74	.27	.80	18	—

denervation itself caused in 2 out of 3 expts. a small enhancement of RPF_{tot} (cf Table I in Nissen 1968) n one of these (exp 7) the deep extraction fraction de clined concomitantly

Loading with *mann tol* solutions resulted n similar changes in the extraction f ac tions as Ringer or Plasma administration (exp 2, 3 Tabl 1) In exp 1 insignificant alterations occurred.

The average superficial extraction fraction during fluid loading rrespectiv of its natur) was 0.86 the deep extraction fraction was 0 64

O le ion f th u t brought about a fall in the uperficial ext ction fractions as well as in the deep fractions (exp I II and III T bl II In the control periods the extraction fractions were rather low presumabl d e t th saline load ng

Th perm bilit of k al er k ec memb P AH
1 1 3 expt the PAH oncentrations of the red cell pon ma nter nc he pla ma concentrations approximat el 22 17 and 16 μg/ml during 4 3 and 3 1 6 10 and 10 of he rterial plasma concentrations 1 2 an n expe oncentrations of sul pula enous blood wer compared ith hose of si lected arterial blood The enous ell concentr tions constituted f ar ore of first experiment and 18 1 and 1 1 second experiment) of the arterial ll on The plasma oncent 29 and 23 μg/ml (first experiment and 14 1 and 1/0 μg/ml second experiment The ell concentrations ere 7 and 9 per ent d 13 1 and 1 ent respec el of he p asma oncentrations

2. I 3 ... expts. no measurable amount of PAH had diffused into the erythrocytes following 2 hrs of incubation in PAH containing plasma, as indicated by the fact that no alteration in the plasma PAH/T 1824 concentration ratio could be demonstrated within the period. The hematocrit values of the samples were 40, 41 and 55 per cent, and the (initial) plasma concentrations of PAH 47 50 and 59 µg/ml, respectively. The last experiment was extended for further 6 hours the plasma concentration decreased steadily in this period to reach value of about 77 per cent of the initial concentration. If PAH had achieved concentration equilibrium across the erythrocyte membranes in this experiment the plasma concentration should have been $100 \cdot \frac{100-55}{(100-55)+0.6 \cdot 55} = 58$ per cent of the initial concentration presuming fractional space of distribution the erythrocytes equal to that of water (0.6).

Discussion

The renal extraction fraction of PAH has always been found to be lower than unity. Trueta *et al.* (1947 p. 130—131) advocated the idea, that the incompleteness of the extraction was the result of the perfusion of medullary tissue. Based on this idea Reubi (1958) introduced a calculation of the medullary plasma flow as the difference between the total renal plasma flow (Cl_{PAH}/extraction $fraction_{PAH}$) and the clearance of PAH (Cl_{PAH}) thus postulating that the cortical and medullary plasma supplies respectively were 100 per cent and 0 per cent cleared of PAH. Inasmuch as the medullary plasma flow is not accessible for direct measurements, the approach of Reubi (1958) has raised considerable interest as apparent from several recent papers (Elpers and Selkurt 1963, Pilkington *et al.* 1965, Hartung and Bartha 1966, Shuster *et al.* 1966). However the validity of the underlying assumptions have never been experimentally substantiated. As to the idea of zero extraction of the plasma traversing the medulla the correctness may in fact be doubted on anatomical grounds. Thus it was argued by Smith (1951 p. 832) that all blood destined for the medulla passes the capillary descending vasae rectae to be exposed to the partes rectae segments of the outer band of the outer zone which were assumed to be capable of PAH secretion.

The existence of two venous drainage routes in the cat kidney allowing collection of venous blood originating from the outer regions of the cortex offers an opportunity to test the validity of the first assumption complete extraction of the plasma traversing cortical areas. A condition for this is, however that uncleared blood from the fibrous capsule plus surrounding fat does not drain to the superficial venous system to be sampled by the catheter. This source of error is of no importance. The fibrous capsule can be readily stripped off and the subcapsular veins show no leakages if they are filled with a suspension of Indian ink through the renal vein, showing that no venous connections were torn by the stripping. In some of the experiments all fat was loosened from the kidney surface without any increase in the superficial extraction fraction. In addition the extraction fractions during heavy hydration were far too low to be accounted for by any conceivable admixture of unextracted blood from the capsules.

A second potential source of error is that the PAH preparations contain impurities, which develop colour in the PAH analysis, but are handled differently from PAH by the kidney. Fluka's and Koch-Light's PAH preparations contained a substance

probably p-aminobenzoic acid, in amounts developing extinctions of some 0.2 and 1.2 per cent, respectively of those of the PAH preparations (Frederiksen and Nissen 1968). However since the extraction fractions were found not to differ significantly in experiments where similar plasma PAH concentrations (below 40 μg/ml) were established by infusion of the two preparations, the 5 per cent incompleteness of the extraction in the superficial area cannot have been due to p-aminobenzoic acid contamination of the preparations. It is improbable that the same p-aminobenzoate concentrations in arterial plasma should have been attained by accumulation, by infusion of p-aminobenzoate at rates differing by a factor of 6 (1.2/0.2). The reason why the plasma concentrations of p-aminobenzoate remain too low to affect the extraction fractions is probably that p-aminobenzoate is converted into PAH in the liver.

Both preparations included also an unidentified substance constituting 0.1 per cent expressed as PAH. A simple reasoning shows that an impurity of this magnitude cannot account for the observed 5 per cent incompleteness of extraction in the superficial area. Thus if the volume of distribution of PAH is 40 per cent of the body weight and that of the impurity is only 5 per cent (the plasma volume) a priming dose of the PAH preparations would confer to the plasma an impurity concentration amounting to 0.8 per cent of that of PAH. To maintain the arterial plasma PAH concentration, as much of the PAH preparation must subsequently be infused per min as is contained in the volume of plasma cleared of PAH per min. This volume amounts to about 1/5 of the total plasma volume. If it is now further assumed that the impurity is not eliminated by the organism an infusion of the preparation at this rate would raise the impurity concentration in plasma by (1/5) 0.1 = 0.02 per cent of that of PAH per min. It would thus take (5—0.8)/0.02 min or 3 1/2 hrs for the concentration of th impurity to reach 5 per cent of that of PAH. The time required would be longer if the impurity ere distributed in a larger olume or were eliminated. Actually the observed extraction fractions were found to remain unchanged during a period extending from 1 to 2 1/2 hrs from the start of the infusion. In 4 animals, kept under as consta t conditions as possible, the average extraction fractions for the superficial area after 1, 1 3/4 and 2 1/2 hrs were 0.930, 0.933 and 0.949 respectivel. Thu there was no sign that an impurity by accumulation affected the extraction fraction.

A third source of error is that signific nt mounts of PAH might leak from the red cells during the tim the cell lea th kidney till the plasma is separated from them. A direct comparison between the PAH concentrations in the arterial and the renal venous red cells is rather ncertai due to the difficulty of determining exact the low PAH concentrations the hemolyzed cell suspension, and due to the that the correction for PAH trapped between the centrifuged cells is much in the case of rterial than in the case of venous blood. However within the ac of the method no differenc between the cell concentrations could be demon That errors c sed by diffusion of PAH from red cells to plasma are of tance is also indica ed by the find ng of a ery low rate of permeatio

red cell membranes in the *in vitro* experiments (the time between collection of the arterial and the renal venous blood samples, and the centrifugation, never exceeded 15 min in the extraction experiments) A low permeability to PAH of the red cell membrane was also found by Eggleton and Habib (1949) In addition human erythrocytes show this feature (Smith *et al* 1945) In dogs the magnitude of the transfer rate is larger and demands a negative correction of the venous plasma concentrations (Phillips *et al.* 1946)

Consequently the observation that the extraction in the superficial cortex is only 93 per cent complete must be correct *i.e* the first premise of Reubi's hypothesis, that the extraction of plasma traversing cortical tissue, is complete is false One possible explanation for this might be that the velocity constant for binding of PAH to a cellular transport component is to low to allow all PAH entering with the arterial blood to be bound to this component within the capillary transit time (Kiil 1961) The velocity of dissociation of PAH from the plasma protein must also be considered, however as a factor which might limit the extraction.

The extraction percentage of PAH in the deep area was lower about 82 It is reasonable to associate this finding with the dominance of medullary tissue in the area. On the other hand Reubi's second premise that the extraction percentage of plasma perfusing the medulla is zero is improbable. It might be positive according to Smith's argument referred to above, or it might be negative owing to the occurrence of back-diffusion of PAH in medullary tubule segments, suggested by the finding of PAH concentrations in vasa recta plasma, collected in the papilla of the golden hamster which ar 4—13 times higher than those of the arterial plasma concentrations (Schnermann and Thurau 1965)

It has been repeatedly shown, that fluid loading reduces the extraction of PAH (Cargill 1948 Michie *et al* 1951 Harth, Kreienberg and Lutz 1959 Braun and Lilienfield 1963 Elpers and Selkurt 1963 Pilkington *et al* 1965) A commonly accepted view is that th reduction in the extraction is caused by the opening up of ch nnels by passing actively secreting tubules (Cargill 1948 Michie *et al* 1951 Elpers and Selk rt 1963 Pilkington *et al.* 1965) The last two groups of authors suggested that the by-pass flow occurs along medullary vessels and calculated it *using the Reubi method Harvey (1966) pointed out that this approach yields* medullary flows which are very high compared to those determined by other methods As demonstrated in the present experiments fluid loading generally reduces the extraction fraction in the superficial as well as in the deep area thus the alterations in the medullary blood flow calculated according to Reubi are highly inexact.

However th extr ctio n fractions usually declined more in the deep drainage area than in the superf cial pon fl id loading While the present study invalidate the Reubi procedure for lc lat on of the medullary plasma flow the possibility till remains that a preferential rise f the medullary blood flow takes place during the loading These problems were discussed in nother paper on the changes of the filtration fractions in the tw drainage reas associated with fluid loading (Nissen 1968)

The fall in the overall extraction accompanying occlusion of the ureter cannot solely be attributed to a diversion of blood through medullary vessels as suggested by Selkurt (1963) because the extraction fractions also declined in both drainage areas during this manoeuvre.

In conclusion it may be stated that the marked fall in the overall and in the deep extraction fraction of PAH elicited by fluid loading may well to a large extent be due to diversion of blood through medullary vessels. However the fact that a simultaneous, although lesser fall occurs in the extraction in th superficial area—even if the overall PAH load is unchanged or is reduced—shows that other factors are involved in the fall in overall extraction, and suggests that they may be operating too in the deep area. The nature of these additional factors can, at present, only be guessed at (increased PAH diffusion pathways due to renal oedema, reduction in cellular secretory capacity increased tubular back-leakage of PAH)

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The Purity of Two Commercial p-aminohippurate Preparations

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Abstract

FREDERIKSEN S. and O I NISSEN. *The purity of two commercial p-aminohippurate preparations.* Acta physiol. scand. 1968. 73 339—340.

P-aminohippuric acid (PAH) preparations made by "Koch-Light" and Fluka were analyzed by column chromatographic technique using columns of Dowex 1-Cl 200—400 mesh, X2 and Dowex 50-H 200—400 mesh, X12. A commonly used procedure for determination of PAH in plasma and urine was applied to the effluents. The preparations contained impurities giving positive PAH reaction in concentrations of 1.3 % (Koch-Light) and 0.5 % (Fluka) expressed as PAH. The major parts of the contaminants are probably p-aminobenzoic acid 1.2 % and 0.2 % respectively.

Nissen (1968) observed that the extraction of p-aminohippuric acid (PAH) in the outer cortex of the cat kidney was only 95 per cent complete even at arterial plasma concentrations below 40 µg ml. Since any impurity in the PAH preparations capable of reacting in the PAH analysis but not being handled identically with PAH by the kidney might be responsible for the incompleteness in extraction, an investigation of the purity of the PAH preparations has been made.

The PAH (from Koch-Light, England, and Fluka, Switzerland, both pure) were analyzed by a column chromatographic technique. Chromatography on columns of Dowex 1-Cl 200—400 mesh, X2 (h=20 cm, d=1.6 cm): 5.0 ml PAH solution, 1.30 mg/ml adjusted to pH —8 was applied to the top of the column. The solution was allowed to drain to the top of the resin. 5 ml distilled water was added and was allowed to drain to the top of the resin. The column was then eluted with a linear gradient of 200 ml of distilled water (mixer) and 200 ml 2.0 M NaCl (reservoir). The effluent was scanned for material absorbing light at 2537 Å with an LKB Uvicord attachment and collected in 5 ml fractions. The method allowed detection of an amount of contaminant producing an extinction of 0.1 per cent of that of the PAH preparation.

Chromatography on columns of Dowex 50-H 200—400 mesh, X12, (h=16 cm, d=1.6 cm). The chromatography was performed as described above except that water and aqueous solutions were substituted with 0.05 M sodium phosphate buffer pH 6.7.

The procedure of Smith *et al.* 1945 for PAH-analysis was applied to the effluent fractions. Any compound which develops colour by this procedure is described as PAH positive. With the amounts applied on the columns 0.1 per cent of PAH positive contaminant could be determined with certainty.

When PAH was chromatographed on Dowex 1-Cl the major peak absorbing ultraviolet light eluted in fraction 60—75 but a small peak eluted in front of this PAH peak in fraction 54—58. The compound eluting in front of PAH also gave a positive PAH reaction. PAH is prepared from p-nitrobenzoylchloride and glycine. The

of this reaction is then reduced to PAH by means of $SnCl_2 + HCl$ (Martin 1965, p. 1373). A possible contaminant of PAH would be p-aminobenzoic acid. The isolation of p-aminobenzoic acid from a commercial sample of PAH has been recorded by Schreiner, Wesson and Anslow (1949). By mixing p-aminobenzoate and PAH it was found that p-aminobenzoate eluted from the column in front of PAH in the same fractions as the contaminant of the PAH. Determined as PAH positive material the contaminant amounts to 0.2 per cent in Fluka's PAH preparation and 1.2 per cent in Koch Light's PAH preparation. In both cases every third fraction of the entire column effluent was analyzed for PAH positive material. Apart from the PAH peak and the contaminant absorbing ultraviolet light (p-aminobenzoate) no PAH positive material was present.

Chromatography on Dowex 50-H gave the major ultraviolet absorbing material in fraction 42—50 with some tailing into fraction 51—56. A small peak was also seen in fraction 4—6. This minor peak was present in both Koch-Light and Fluka PAH. The compound gave positive PAH reaction and amounts to 0.1 per cent of the PAH, expressed as PAH. Every third fraction of the entire column effluent was analyzed for PAH positive material, but apart from the PAH peak and the unidentified ultraviolet absorbing material in fraction 4—6, no PAH positive material could be detected. The tailing of the PAH peak on this type of column could not be avoided even though the column was eluted very slowly (15 ml per hr). This tail might cover some minor impurities although this seems unlikely because of the smooth form of the tail. By chromatographing the PAH on Dowex 50-Na and thereby operating at less acidic conditions it should be possible to further separate PAH from a possible impurity in this tail. PAH passed almost directly through the Dowex 50-Na column (h = 12.5 cm, d = 1.6 cm) when this was eluted with a gra- ... of 200 ml water and 200 ml 2.0 M NaCl. A few fractions of the latest eluting ...aviolet absorbing material were pooled and the amount of PAH positive material determined. To this solution was added an amount of PAH preparation representing the same extinction and the mixture was re-chromatographed on Dowex 50-H as described above. The mixture chromatographed as one distinct peak containing PAH positive material corresponding to the sum of the materials applied to the column.

In summary it can be said that the PAH preparations used in the experiments contain PAH positi... contaminants in concentrations of 1.3 per cent (Koch-Light) and 0.3 per cent (Fluka). Further that the major parts of the contaminants are probably p-aminobenzoic acid, 1.2 and 0.2 per cent, respectively. Whether the existence of impurities in these concentrations can explain the incompleteness in renal PAH extraction is discussed by Nissen (1968).

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Posthypertonic Hemolysis in Sodium Chloride Systems

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Abstract

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Human erythrocytes were exposed to hypertonic NaCl solutions and then to near isotonic media. The resulting posthypertonic hemolysis was studied as a function of hypertonic concentration and of time in the hypertonic medium. Providing that a critical concentration level (~1.3 M NaCl) is exceeded, posthypertonic hemolysis is proportional both to the duration of exposure to hypertonicity and to the hypertonic concentration. The results have been interpreted in terms of a hypothesis put forward by Söderström (1944) who postulated that influx of salt occurred during the hypertonic phase and that later reduction of the external concentration caused osmotic hemolysis. A rough calculation suggests that only above an external concentration of 0.65 M NaCl will an influx of salt occur. An analysis of the results in terms of this hypothesis suggested that the permeability of the cells to NaCl increases with increasing external NaCl concentration. The permeability increase was very great in the region of 3 M NaCl. The counter-current multiplier mechanism for kidney function has been criticized on the ground that red cells may not tolerate the tonicity changes postulated to exist in the kidney. In vitro experiments using slightly worse conditions than are believed to exist in the human kidney showed no significant hemolysis.

In this paper posthypertonic hemolysis is defined as the hemolysis which occurs when red cells suspended in a medium of high tonicity are transferred to a lower solute concentration (not less than isotonic). The phenomenon was first described by Takei (1921) who used glucose solutions. Söderström (1944) briefly discussed its mechanism, in a study of hemolysis in hypertonic salt media. This type of hemolysis is at present of great interest with regard to *blood preservation* as blood usually hemolyses to some extent when subjected to freezing processes. The degree of hemolysis apparently depends on the increase in salt concentration which occurs when water separates out as ice and also on the time during which the cells are exposed to this hypertonic environment (Lovelock 1953, Valdivieso and Hunter 1961).

The view that solute concentration rather than mechanical trauma is the prime cause of freezing injury is by no means new (Müller-Thurgau 1886). Gorke (1907) showed that some plant proteins were denatured by freeze-thawing and also that high salt concentrations had a similar effect. Kylin (1917) reported that marine

The absorbance was determined at 540 nm or for samples with low Hb concentration, at 420 nm. The absorbance value at 420 nm was divided by 10.17 to convert to the value for 540 nm (Zade-Oppen 1960).

The degree of hemolysis was expressed as the fraction in per cent of the Hb liberated from the cells. The total Hb present was measured in one of two ways.

1. In each experiment four or five initial cell suspensions were diluted with water instead of saline so as to obtain the same dilution as in the other samples but with complete hemolysis. These samples were not centrifuged and the Hb concentration was measured as above; the average absorbance value was used as the 100 per cent reference (A_m). This procedure is possible only if all cell dilutions are known as is the case in MIX P 1. It cannot be used with MIX P 2.

2. 4 ml of the final cell suspension was hemolysed by the addition of about $5\times10^{-}$ ml of ~3 g/l digitonin (solution heated to disperse properly). 0.1 ml of nitrite reagent were then added.

Neglecting the volume of the drop of digitonin solution, the cell suspension was thus diluted to the same extent as the supernatant, and liberated Hb was calculated as $\frac{A_s \cdot 100}{A_m}$ per cent, where A_s is the absorbance of the supernatant and A_m that of the mixed sample. In this way each supernatant had its own 100 per cent reference. This method was used as an additional check in MIX P 1.

With MIX P 2 (5 expts.) the samples had to be diluted before analysis and Hb was determined as follows.

1. The supernatant was diluted 5 times with 40 times dilution of the nitrite reagent.

2. The uncentrifuged sample was diluted with a known amount (~10×) of Drabkin's solution (1 g $NaHCO_3$, 0.052 g KCN and 0.198 g $K_3Fe(CN)_6$, dist. water to 1000 ml). Because of the large amount of Drabkin solution used no additional lysis was required. The calculation was made as above after correction for dilution.

Hb in plasma

In the one experiment where plasma was used as suspending medium, it was necessary to modify the method because of turbidity. To 4 ml supernatant plasma, 0.1 ml 0.8 per cent $K_3Fe(CN)_6$ and 0.1 ml 0.2 per cent KCN (pH adjusted to about 7.5 by careful addition of 0.1 N HCl) were added. This treatment transformed Hb into the MHbCN form. A solution of pure MHbCN was also made from a toluene hemolysate from washed erythrocytes (as described by Zade-Oppen 1963). The plasma MHbCN was then determined by the variable reference technique (Hakes 1955). After the Hb concentration of the reference solution was made equal to the Hb concentration of the unknown sample as judged from the disappearance of the 540 nm peak (Beckman DB Spectrophotometer with Sargent SRL recorder), the Hb concentration of the reference was determined at 540 nm on the same instrument.

Accuracy of dilution

The reproducibility of the volume of packed cells delivered from the 1 ml gas tight syringe was tested by advancing the piston 1 mm to deliver 16.7 µl cells into each of 10 tubes containing 4 ml of a nitrite reagent solution diluted 40 times. The absorbance at 540 nm was determined and was found to have a coefficient of variation of ±0.56 per cent.

The absorbances of the total Hb samples in 12 experiments on posthypertonic systems had an average standard deviation of 1.95 per cent of the mean, ranging between 1.12 per cent (n = 20) and 4.61 per cent (n = 29).

Determination of Na and K

Determination of Na and K in erythrocytes was made with an "Eppendorf" flamephotometer (Netheler and Hinz GMBH, Hamburg) after hemolysis and precipitation of the proteins as according to Overman and Davis (1947). The procedure was slightly modified by using 0.1 per cent NH_4OH for dilution instead of water in order to obtain more complete hemolysis (Barer and Gaffney 195–) and by using a higher degree of dilution, which better suited the instrument used. Calibrations were made in the presence of the corresponding concentrations of protein precipitated.

Results

Time between mixing and centrifugation

In order to assess the degree of hemolysis resulting from transfer to a medium of a lower salt concentration, time must be allowed for essential completion of hemolysis

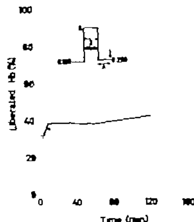


Fig. 1 The time required to reach steady alue of Hb libera ed in posthypertonic hemolysis. Centrifugation was performed t different intervals after the last addition of saline to the sample. The time therefore refers to the interval between mixing and centrifuging samples.
The inset in this and subsequent figures is intended as short description of the experiment and the ordinate represents saline concentration in molarity ith time on the abscissa.

before the centrifugation of the suspension for measurement of liberated Hb in the medium.

An experiment was therefore made in which cells were suspended in 2.0 M N Cl buffer for 2 min before dilution to 0.259 M. The samples ere centrifuged for 20 min at 3000 × g with the intervals between the final mixing and centrif ging arying from ? min to 1?0 min. The result is shown in Fig 1 From 10 min om ards there was no significant change.

Cell Na and K prior to th introdu tio of a hypert nic medium

As human erythrocytes lose K and gain N when kept at room temperature, an experiment was made to find out the magnitude of this exchange during the standard procedure preceding the introduction of cells into a hypertonic medium.

The initial cellular Na and K -concentrations were measured on cell samples obtained from blood which was centrifuged for 15 min at 37,500 × g commencing within 3—5 min after enesection.

Determinations were also made on cells subsequent to the washing procedure Cells sedimented after the third wash (3000 × g ?0 min) were then centrifuged t 37,500 × g for 15 min to minimize the trapped interstitial fluid

Finally the ion concentrations were determined on packed cells which after washing were allowed to tand at room temperature for 1 hr nd were then dil ted 1 + 4 with isotonic saline and again left for 90 min before centrifugation. Duplicate deter minations were made throughout. Analyses were also made on the supernata t fluids to permit correction for trapped plasma.

The result is shown in Table I. Duplicate determinations were in good agreement with each other and the table gives the average alues. The results are given w thout nd with correction for 0 5 per cent (unpublished observation) trapped plasma or saline This result means that the cells may be expected to exchange up to bout 10 mN K and Na prior to the introduction of the cells into the hypertonic medi

TABLE I Na+ and K concentrations in red cells before transfer to hypertonic environment

Trapped plasma	0		0.5 %	
	Na	K	Na	K
Fresh	12.8	106.3	12.2	106.8
After 3 washes	14.3	104.9	13.7	103.4
60+90 min later	21.8	95.9	21.2	96.3

Concentrations in mN per liter packed cells.

In order to avoid any systematic error due to changes arising during the unavoidable storage period before transfer to the hypertonic environment, the storage time was varied as randomly as possible with respect to the other experimental parameters.

Hypertonic Hb liberation

In order to assess the magnitude of posthypertonic hemolysis, it is necessary to know the degree of hemolysis in the hypertonic phase of the experiment. As it is rather difficult to measure the hemolysis occurring during very short times in a hypertonic medium the shortest period studied was 2—3 min.

In 2 expts. 0.25 ml samples of initial cell suspension were diluted with 15 ml of various hypertonic solutions. Parts of these mixtures were spun in an angle head centrifuge which was started not less than 2 or more than 3 min after mixing the individual samples. The centrifuge needed approximately 25 sec to reach 37 000×g, which was then maintained for 5 to 10 min. Other parts of the mixtures were centrifuged 3 hrs after mixing in a swing-out head at 3000×g for 10 (Fig 2) or for 20 min. The two experiments gave essentially similar results except that while the Hb-liberation after 3 hrs at the highest salt concentration was 60 per cent in the one experiment (Fig 2) it was only 43 per cent in the other.

The 2—3 minute samples were centrifuged at 37 500×g in order to accomplish sedimentation and hence removal of supernatant for hemoglobin determination as rapidly as possible. This, however, probably caused increased hemolysis, due most likely to mechanical trauma, since a 2 min curve obtained by centrifugation at 3000 g (Fig 3) showed no hemolysis below about 1.5 M.

The curve of Fig 3 was obtained by diluting 0.25 ml initial cell suspension with 1 ml hypertonic saline (as in MIN P 1) and centrifuging for 5 min in a swing-out head at 3000×g. The centrifuge was started 2 min after mixing and it needed approximately 15 sec to obtain full speed. Quintuplicate samples were run at each salt concentration. A second experiment with duplicate samples gave results which are not significantly different.

After 2 min there was less hemolysis in the lower salt concentrations, at least in the cells centrifuged at the lower speed (swing-out head) than at the higher speed (angle head). Though the final cell dilutions were not the same it is assumed that

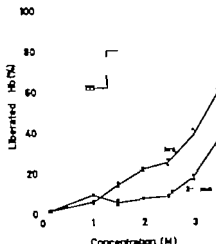


Fig. 2. Hypertonic Hb-liberation in media of different concentrations. Hemoglobin was measured in the supernatant after centrifugation at 37 000 × g, which was started 2 to 3 min after mixing or after centrifugation at 3000 × g 3 hrs after mixing

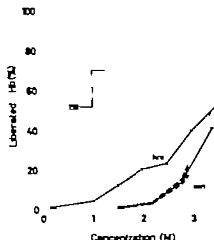


Fig. 3. Hypertonic Hb liberation in media of different concentrations as measured 1) (○) after centrifuging during 5 min at 3000 × g was started 2 min after mixing. The full line combines the averages of 5 samples for each point. Dashed lines for ±2 standard errors of estimate. 2) ( ) after centrifuging at 3000 × g was started 3 hrs after mixing. Mean of 2 expts

this did not contribute to the difference in hemolysis. Since hypertonic hemolysis increases with time (see Fig 2 and 3 and Söderström 1944) the finding of more hemolysis after the more rapid sedimentation probably points t mechanical trauma as responsible for the difference. This appears to disappear t higher salt concentrations.

To analyse correctly the following experiments on posthypertonic hemolysis the hypertonic Hb liberation should be subtracted from the figures given T do this the curves hown here may serve as n indicator of the magnitud

K th m dium

Some of the experiments (see methods) were made without K in the mediu Fig 5 show one double-determination in a K-free medium which is similar to the cor

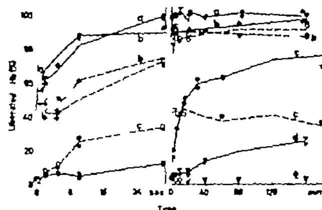


Fig 4 Posthypertonic Hb liberation after different times at 4 different hypertonic concentrations.
The left part of the figure represents the first 30 sec of some of the experiments on an expanded time scale. Whole lines indicate that mixing procedure I (MIX P 1) was used, and dashed lines that the inferior MIX P was used.
The result obtained with fresh blood (curve a, whole line) is very similar to that obtained after storage of the same blood for 6 days at +5 C in ACD-solution (curve b, whole line)

Comparison of the results obtained with different concentrations in the hypertonic phase of the experiments show that the resulting posthypertonic hemolysis increases with the degree of hypertonicity and with the time of contact with the hypertonic environment.
Comparison of the dashed lines (MIX P 2) with corresponding whole lines (MIX P 1) show that the use of MIX P 2 may give misleading results.

Concentrations used (M)

Curve	a, b		d	
Hypertonic concentration	3.025	1 530	1 318	0 672
Posthypertonic concentration	0 317	0.234	0.280	0 210

responding points with K The material is too small to be conclusive but indicates that this difference is probably not important. It may be noted that curve c (whole line) of Fig 4 without K at 30 min lies above the range of the curves of Fig 5 with K in this case however a slightly different mixing technique may be responsible This was not, however investigated further

Background hemolysis

There was usually a little hemolysis in samples which were subjected to the same mixing and centrifugation procedures as in the hypertonic systems but brought into contact with isotonic saline only (see for example points at zero time in Fig 4 and points at isotonic concentrations in Fig 5 and 6)

The effect of the time of stay in the hypertonic medium

Fig. 4 shows results of cells kept for varying times in different hypertonic solutions. After exposure to 3.025 M for as little as 1 or 2 sec, there was a high degree of Hb liberation after reduction of the tonicity to a near isotonic level 30 sec at 3 025 M were sufficient to produce complete hemolysis after reduction of the saline concentration.

The degree of posthypertonic hemolysis appears to increase with both the degree of and the time of exposure to hypertonicity ("hypertonic time") The initial slope

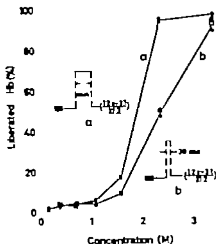


Fig. 5 Posthypertonic Hb liberation after different peak concentrations maintained during min (filled circles) or 30 sec (open circles) The tw curves were obtained from different experiments. The 2 min experiment included 2 points (half-filled circles) which were kept at the peak concentration during only 30 sec to allow comparison with the other curve.
Triangles represent samples containing 0 667 M urea in the hypertonic phase Electrolyte concentration on the abscissa. Squares represent samples ithout K in the media, in contrast to the other points of the same graph.

of the degree of hemolysis as a function of hypertonic time" ary directly with the hypertonic level (see Fig 4) There also appears to be a tendency to reach a maximal degree of hemolysis (when this is less than 100 per cent) which also increases with hypertonicity (Fig 4)

As can be seen in Fig. 4 curve a, whole line the maximal level of hemolysis is nearly reached after 30 sec but the process takes longer at lower h pertonicities (Fig. 4 c and d)

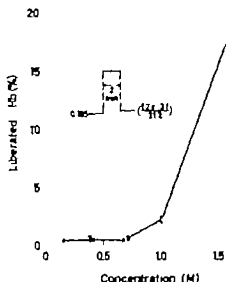
Eff ct of different levels of hypert icity n posthypert c Hb liberation

As posthypertonic Hb liberation depends on both the time of exposure to, and on the degree of hypertonicity a very large number of experiments would be necessary to analyse the process completely In experiments in which hypertonic salt concentrations were aried, only times of 30 sec and 2 min (Fig. 5) were therefore selected.

This choice, which may appear somewhat arbitrary was made because these studies were originally initiated with regard to the beha iour of red cells in transient hypertonic environments such as occur during freezing and during their passage through the kidney

The 30 sec and 2 min experiments were done on diff rent days. In order to enable a comparison to be made the min experiment also contained two blood samples which were exposed to 2.351 M for 30 sec only this concentration being selected because t ga bout 50 per cent hemolysis at 30 sec As ma be seen in Fig. 5 there was a rather good fit between these two points nd th rresponding ones in the 30 sec experiment. Th figur also shows that th min rv s steeper and that the two curves di erge after seemingly arising at bout th same molarity i.e. about 0.8 M

This does not necessaril mean that l curves meas red after ery long times at the hypertonic level there is not a shift of the origin towards lower concentrations Such shift cannot be large however ince t 0.67? M there was no Hb liberati



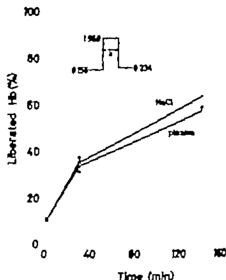


Fig 6	Fig 7

Fig 6 Posthypertonic Hb liberation after different high peak concentrations maintained during 2 min. The open circles represent samples containing 0.8 M rea in the hypertonic phase Electrolyte concentration on the abscissa.

Fig 7 Posthypertonic Hb liberation after different times at a hypertonic concentration of 1.550 M saline (open ircles) and plasma (filled circles) were used as media. The overlap of duplicate determinations from the two curves means that they are probably not significantly different.

after expos re for as long as 160 min (Fig. 4 e) Both the 30 sec and 2 min curves exhibit slight increase in hemolysis at low molarities. This may not necessarily be due to any posthypertonic hemolysis but to the mechanical treatment of the cells uring the fast mixing procedure cells in a hypertonic med um being somewhat more sensitive to mechanical trauma. An attempt was therefore made to reduce the mechanical trauma by mixing in wider tubes and adding the solutions in a somewhat gentler manne The m xing time was thereby slightly prolonged but could still be regarded negligible in comparison with the time in the hypertonic medium. As shown in Fig 6 there is no hemolysis below 0 72 M

Effect of th add tion of a

In three experiments, urea was added to the hypertonic solutions to gi e concentrations of 0.667 M and 0 8 M n the hypertonic suspensions (see Fig 5 and 6) In all cases, the peak lectr l c ncentrations were 0.384 or 0.718 M The Hb liberation was not significantly lt rd

Posthypertonic Hb lib t plasma and salin

The isotonic saline used in the bov experiments is an artificial medium for the red cells with a composition ery d fferent from their natural medium. In order to assess the possible significance of th bsence of a number of natural components in the

medium such as glucose, protein, phospholipids, Ca^{2+} Mg^{2+} an experiment was performed in which part of the cells were washed and treated as usual before exposure to the hypertonic system. A second part of the same blood was treated as follows.

Blood was centrifuged 20 min at 5000×g The plasma was then removed and part of it was made hypertonic by adding dry NaCl. The plasma was then equilibrated with room air The pH of both the hyper and isotonic plasma was adjusted to 7 40 (the same value as that of the saline solutions) by addition of N HCl, which thus substituted for the CO_2 lost. Th initial cell suspension was then made by diluting the packed cells with isotonic plasma. Hypertonic, and later isotonic plasma were added in the same way as with the reference saline series. The plasma series had its own 100 per cent Hb reference where water was added instead of hyper and isotonic plasma. The plasma was very turbid, and the usual procedure for the determination of liberated Hb was not therefore reliable (see methods)

No significant differences were found between the two series (Fig 7)

Individual variations

A number of experiments were made under similar conditions with blood from different blood donors. The age of the donors varied from 17 to 46 years (the age and sex of one donor were not available) All the bloods were taken at about the same time of day from apparently healthy subjects (most being students) Fig. 8 shows a relatively large scatter of hemolysis after exposure to 2 M for 2 min, which is not unexpected because of the steepness of the curve at this concentration. The scatter at 1.55 and 2.5 M is smaller In Fig 9 which shows the posthypertonic hemolysis after different times at 1.550 M the differences at 80 min for example are rather large the values varying from 45 to 78 per cent.

By analyses of variances using th component of variance (random effects) model (Ostle 1963) it was shown that the scatter was due also to differences between the experiments and not only to the error of the method (Table II) This statistical model assumes that both th uncertainties in the method and the differences between experiments are random Variations between different experiments are very significant ($P>0.995$) except at 140 min (Fig 9) where $0.90<P<0.95$ and at 1.55 M/2 min of Fig 8 where $0.975<P<0.990$ (Th difference between the 1.55 M/2 min points of Fig. 8 and Fig 9 is due to the larger number of experiments Fig. 9 (see Table II))

The whole line experimental curve of Fig. 4 seems in part to have a significantly different shape than the other figures The 30 min point at least is too high The only experimental difference between this experiment and the others is that K free solutions and narrower test tubes were used with a higher degree of mechanical trauma in the case shown in Fig. 4

Comparison of the two different mixing techniques

Fig. 4 shows that somewhat divergent results were obtained with the two different mixing techniques MIX P (Lovelock technique) showed less liberation in

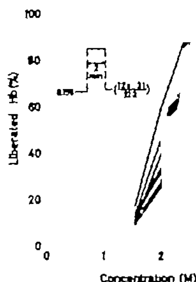


Fig. 8

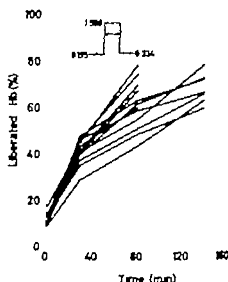


Fig. 9

Fig. 8. Posthypertonic Hb liberation after different hypertonic concentrations maintained during 2 min. 10 experiments with blood from different donors.

Fig. 9. Posthypertonic Hb liberation after different times at a hypertonic concentration of 1.550 M. 12 experiments with blood from different donors.

TABLE II

Fig	8			9			
Hypertonic conc. M	2.5	2.0	1.55	1.55	1.55	1.55	1.55
Time min	2	2	2	2	30	80	140
[illegible]	1.27	2.23	1.55	1.39	2.81	4.55	3.16
	2.16	10.11	2.45	2.37	5.08	9.94	3.23

The table shows the scatter among the values given in Fig. 8 and 9.
s = Estimated standard deviation of the method.
σ = Estimated standard deviation between experiments.
The 10 expts. which are represented by the 1.55 M point in Fig. 8 are also included in the 12 expts. of the 2 min. point in Fig. 9.

experiments and more in one after short times in the hypertonic medium. After a long time it gave less liberation in all three experiments. From the shape of the curve no conclusions can be drawn as to which technique gives the most correct result. However the poor mixing of cells in the hypertonic medium and the incomplete emptying of the contents of the pasteur pipette in which small lumps of packed cells often remained, means that MIX P 2 must be suspected of giving rather misleading results.

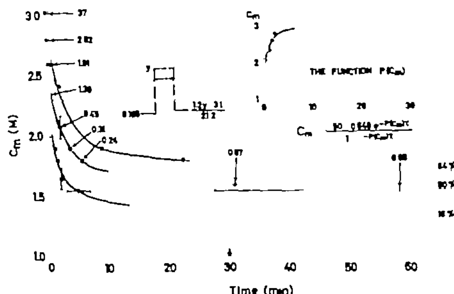


Fig. 10. The relationship between concentration and time in the hypertonic medium required to produce 16, 50 and 84 per cent posthypertonic hemolysis. The values along the 50 per cent line give the values of P required to fit this line to equation 2. The relation of these P values and the external salt concentration (C_m) are also shown in the inset graph.
The experimental conditions are given in an inset with y = external salt concentration and = time.

Comparison between fresh and stored blood

Fig. 4 a and b shows results from experiments made after different storage times on the same blood. The first experiment was made on fresh blood taken in a dry tube containing heparin while the second one was made on blood taken simultaneously in Acrdex solution and stored for 6 days at +5 °C before washing and using the red cells. No significant differences were found.

Equihemolysis curves

Fig. 10 has been compiled from all the experiments (irrespectively of whether they were done with a fixed time and varied salt concentration or *vice versa*) and shows the immersion time in the hypertonic medium required to produce a posthypertonic hemolysis of 50 per cent (the 50 per cent points have been calculated in most cases by graphic interpolation) plotted as a function of the concentration of the hypertonic medium. The graph shows that the relationship between the time and concentration is nearly hyperbolic. 1.55 M is the lowest experimental concentration which gave 50 per cent posthypertonic hemolysis. At higher concentrations the hypertonic time required to give 50 per cent hemolysis is very short (1 sec at 3.03 M).

The curves for 16 and 84 per cent hemolysis are also given. These values were



Fig. 11 View in phase contrast of cell suspension after partial posthypertonic hemolysis

About 50 µl washed and packed cells were added to 0.1 ml of a 2.25 M solution. Two min later the suspension was diluted with 2 ml of an isotonic solution.

The picture shows two types of cells "Ghosts" which have a very low protein content as judged from their low contrast and bright cells with seemingly normal contrast and protein content.

chosen on the assumption that the hemolysis curve of the population has a Gaussian distribution and therefore represents ± one standard deviation. The concentration difference between the curves appears roughly constant though perhaps increasing with increasing concentration.

Discussion

Background h molysis

The background hemolysis is probably due to mechanical damage to the cells. For example when the tubes containing the cells were narrower the background hemoly sis as higher (Figs. 4 and 5) When wider tubes were used, mixing could be achieved less violently and with less foam formation. In the first part of the curves of g 5 (narrow tubes) there is a slight rise in hemolysis from 2 per cent at isotonicity 5 per cent after 0 672 M In Fig 6 (wider tubes) hemolysis was constant (0.5– per cent) up to an electrol te concentration of 0 718 M

Lovelock 1953 howed that there is more posthypertonic hemolysis if the cells are centrif ged in the hypertonic environment and that this is related to some extent to the centrif gal force used This was interpreted as indicating increased sensitivity of the cell t wards "mechanical shock" in hypertonic media. In the present study some experiment were made with wider tubes in order to reduce mechanical disturbances. No attempt was made to investigate the contribution of mechanical trauma after exp re to concentrations high enough to result in posthypertonic hemolysis. If it is of the same order of size as at concentrations which do not produce posthypertonic hemol or even a little larger t can be neglected in view of the great variations betwee periments.

Hypertonic hemolysis

The hemolysis of red cells in ery hypertonic media was described by Hamburger (1907) Söderstrom (1944 studied the phenomenon in considerable detail.

In the present work, the combined effect of hypertonic and posthypertonic hemolysis has been studied. To determine the degree of posthypertonic hemolysis alone, the contribution of hypertonic hemolysis must be known. Since the latter was found to be rather small, it was subsequently neglected and no correction was made for it. The magnitude of this hemolysis can be assessed from the experiments on hypertonic hemolysis (Fig. 2 and 3). Centrifugation at high speed in an angle head produced more hemolysis than with moderate speed in a swing-out head in spite of the longer duration of the latter procedure. This difference was presumably due to mechanical factors to which the hypertonically shrunk cells seem more sensitive. Greatly shrunken cells may differ in their mechanical rigidity from normal cells. The trapped saline volume was estimated as 16 per cent of the cell fraction in cells centrifuged at 37,500×g in 1.1 M NaCl (Zade-Oppen, unpublished). Further, the hemoglobin concentration in the normal cell is about maximal for a true solution (Perutz 1948). It is also evident in other red cells where hemoglobin is not apparently in true solution, i.e. paracrystalline rat red cells and sickled cells (Ponder 1948) that the fragility is greatly increased. That hemolysis occurs therefore even after moderate centrifugation speeds is perhaps not surprising. The 3 hr curve (Fig. 2 and 3) obtained with 3000×g is in good agreement with the data of Söderström (obtained after spontaneous sedimentation). Thus the mechanical hemolysis in the 2 min experiment of Fig. 3 should not be of significant magnitude.

Posthypertonic hemolysis

The results presented here show that posthypertonic hemolysis is a function of both the duration of exposure to, and the concentration of, the hypertonic medium, providing the latter exceeds a certain critical level.

Since the red cells exhibit rather wide individual variations, several of the results presented here do not represent the behaviour of the average cell. This is perhaps represented best by the 50 per cent hemolysis point. Use of the 50 per cent point as a mean value is, however, only valid if the hemolytic process in the individual cell approximates to an all-or-none process. This assumption appears to be correct since observations under phase contrast show that a partially hemolysed suspension consists essentially of two cell types, one with a high refractive index and the other with a low refractive index (ghosts) (Fig. 11). Fig. 10 shows the time in the hypertonic solution required to produce a posthypertonic hemolysis of 50 per cent as a function of the hypertonic concentration. 1.55 M thus appears to be at or near a critical level below which the "mean" cell does not hemolyse. The curve of Fig. 10 applies, of course, only to the conditions used in the experiments in which the cells were not restored to isotonicity but were transferred to another medium hypertonic, whose concentration was proportional to the hypertonic level. The boundary line might therefore be lower than 1. [illegible] M if [illegible] were [illegible]. If hemolysis were a linear function of the [illegible] the [illegible] would be about 1.47 M. The [illegible] can also be estimated from Fig. [illegible] by extrapolation of the [illegible] of the [illegible] hemolysis [illegible] both graphs the

intercept ... this value is however somewhat uncertain because of the few experimental points.

1.55 M was the lowest concentration after which 50 per cent hemolysis was obtained, but the critical concentration may be below this level. The next lower level studied, 1.318 M, gave only ... per cent hemolysis after 160 min. If it is assumed that even with longer exposure times the latter level will not yield 50 per cent hemolysis, then the critical concentration must lie between 1.55 M and 1.318 M. In view of the shape of the curve in Fig. 10 1.5 M has been taken as the critical value.

Variations in the cell population

Fig. 10 shows the curves for 16, 50 and 84 per cent hemolysis. The distance between the curves is a measure of the variation in the cell population, assuming that hemolysis is a quasi all-or none process for the individual cell.

The values in the figure are not corrected for hypertonic hemolysis. Such a correction would result in a small upward shift (increase in molarity) greatest in the 16 per cent and least in the 84 per cent curve. It is doubtful whether the dissymmetry in the distances between the 16 per cent and 84 per cent curves and the 50 per cent curve is significant.

Comparison with Lovelock data

Lovelock (1953) described the quantitative effects in posthypertonic hemolysis when the time in the hypertonic medium varied up to 60 sec and the degree of hypertonicity varied up to 4 M. He concluded that "the rate of destruction by strong salt solution is seen to be similar to that which occurs on freezing and thawing". He also concluded "that the exposure of red blood-cells to salt solutions stronger than 0.8 M is damaging". He made, however, no further statements concerning the time and concentration scales, and his conclusion concerning the critical concentration of 0.8 M was drawn from an experiment of hemolysis due to sudden cooling at a constant NaCl concentration and not from experiments on posthypertonic hemolysis.

Lovelock's quantitative results appear to contain some contradictions. Judging by the ... Table III (Lovelock 1953) hemolysis has already attained a maximal degree ... 10 sec in a hypertonic medium. In 3 M and 4 M solutions he obtained 64 per cent and 84 per cent hemolysis respectively, maximal time in hypertonic solution ... In Table II he shows however the same hemolysis in 2.5 M, 3 M and 4 M, 80, 81 and 8... per cent respectively after 5 min.

An essential difference between the data presented here and those of Lovelock is the duration of hypertonicity required to ... maximal hemolysis. Lovelock reports, for example, in a ... solution after 10 ... per cent hemolysis after 30 sec and 60 sec, 11 per cent ... results ... increasing hemolysis up to 80—140 min hypertonicity (e... in Fig. 9).

Another differ... Lovelock ... M NaCl a maximal hemolysis of only 80 and 81 ... 60 sec and ... respective ... Table II and III ... but in one case ... 100 per ... hemolysis ... obtained in 3.6 M ...

time being given. In the present study 100 per cent hemolysis was obtained after 30 sec in 3.025 M (Fig. 4) 92 per cent after the same time in 3.384 M (Fig 5) 94 per cent after 2 min in 2.351 M and 97 per cent after 3.384 M (Fig 5)

The explanation for these differences and the discrepancies in Lovelock s results may be the differences in mixing techniques and the disad antages (Harboe 1959) of the hemoglobin determination method (oxyhemoglobin at alkaline pH) used by Lovelock.

The minimal co centration for posthypertonic hemolysis

From a practical point of view it would be of interest to be able to determine a minimal concentration level, below which posthypertonic hemolysis does not occur From experiments on thermal shock" Lovelock (1953) concluded that 0 8 M NaCl was the minimal concentration. S nce however hemolysis in a red cell suspension is a process involving a large number of cells, it is probably not meaningful to attempt to define within close limits a starting point, i.e the point where hemolysis is just above zero. It is, however possible to indicate a concentration range within which hemolysis begins in th cell population. Fig. 6 shows that after suspension in 1.022 M for 2 min, 2.1 per cent hemolysis occurs which is a value significantly higher than that after 0.72 M. It is, of course, quite likely that with a longer hypertonic period hemolysis might also be higher at the lower salt concentration. If this occurs, t must be to a limited extent, however Fig 4 c shows that after suspension in 0 672 M for 160 min there is no subsequent posthypertonic hemolysis. It is perhaps of interest in this connection that cells in this concentration region are maximally shrunken (Takei 1921 Penati and Donati 1938)

Mechanism of posthypert ni hemolysis

Dehydration (Moran 1929) an osmotic phenomenon (paradoxical hypotonic hemolysis) (Söderström 1944) the lytropic properties of lectrolytes (Lovelock 1953) denaturation of cell-lipid protein complexes accompanied by permeability changes (Lovelock 1957) and shrinkage in solutes (Valdiviezo and Hunter 1961) these have all been postulated to account for the cell damage which occurs as a result of exposure to transient hypertonicity

According to Lev tt (1958) Lovelock s own data contradict his conclusions that posthypertonic hemolysis is the cause of hemolysis after freezing. This criticism is based on the fact that hypert nic hemolysis is much less than the hemolysis obtained after freezing (with subsequent thawing)

Lovelock (1957) states "The loss of phospholipid renders the cell membrane permeable to cations so that it slowly swells and ultimately bursts. The transfer of cell engorged with cations back to physiological saline as would occu on thawing uses its immediate lysis In the first sentence a possible mechanism of hypert nic hemolysis is postulated in the second cause of posthypertonic hemolysis. It must however be emphasized that it has not been established whether the two phenomena are basically the same as pparently suggested by Lovelock. The engor ement

hypothesis is similar to that made earlier by Söderström (1944) and should also be valid for non-electrolytes. Posthypertonic hemolysis also occurs after exposure to hypertonic glucose or sucrose (Takei 1921 Valdivieso and Hunter 1961) As pointed out by Söderström (1944) it is a necessary assumption in this hypothesis that the cell membrane must continue to function as a barrier When the external concentration is reduced, water moves into the cell much faster than solutes can escape.

The theory of paradoxical hypotonic hemolysis put forward by Söderström (1944) seems to fit the data best and has been adopted as the working hypothesis. As Söderström outlined his theory rather briefly it will be considered in more detail here.

The normal red cell can attain osmotic equilibrium with its environment over a fairly wide concentration range (Williams et al. 1959) In this case the rapid water exchange (Sidel and Solomon 1957) quickly reduces any solute gradient which might act as a driving force for net solute movements. The cell has not of course, an unlimited capacity to change its volume. In hypotonic solutions it hemolyses when it reaches some critical volume. As the external concentration increases the cell volume decreases and the internal salt concentration rises. The cell is known to shrink to such an extent that it is unlikely that the hemoglobin remains in true solution. Whether the cell has a true minimal volume is not known. In view of the lack of knowledge about the state of its interior in a greatly shrunken state its osmotic behaviour cannot be predicted with any certainty It seems not unlikely provided other events, such as cell damage, do not supervene, that the volume will approach a limit asymptotically at high external concentrations. An important consequence of this virtual minimal volume is that there is also a virtual limit to which the internal salt concentration can be raised. At high external salt concentrations there will be thus, after cessation of net water movements, a gradient which can drive salt into the cell

When the cell is returned to an isotonic medium, water is again quickly taken up solutes permeate much more slowly than water the cell will swell to a volume which will depend on its solute content. If the critical volume is reached in this process then hemolysis occurs (Ponder 1948)

Since the cell must take up a certain amount of salt in order to swell subsequently to its critical volume there must be a critical external solute concentration which is just sufficient (after a long contact time) to provide the cell with the salt increment it requires to reach the critical volume when returned to an isotonic environment. This critical concentration (for the average cell) should be higher than that just required to reduce the volume to a virtual minimum

Quantitative aspect

a) *Interpretations of experimental results* The lowest external solute concentration which shrinks the cell to its virtual minimum was calculated as 0.65 M NaCl. This value was calculated under the following assumptions.

1 At or below this external solute concentration there is no net solute influx into the cell.

2. At the virtual minimal volume the hemoglobin concentration has risen from 34 to a maximum possible value of 64 g/100 ml cells. The latter figure is in fact the concentration of hemoglobin in a hemoglobin crystal (Perutz 1946).
3. There are 0.3 g non-solvent water per g hemoglobin, as in a hemoglobin crystal (Perutz 1946) and in a 20 per cent Hb solution (Schwan 1965). The value is independent of Hb concentration (8—24 per cent) and similar for red cells and Hb solutions (Gary Bobo 1964).
4. The water content of the normal cell is 0.72 ml per ml cells (Savitz, Sidel and Solomon 1964).
5. Activities are assumed to equal the concentrations.

The shrunken cell volume would thus be calculated as 0.53 of the normal value which is by no means an unreasonable value. Takei (1971) obtained a minimal hematocrit value of 0.64 but no correction was made for trapped plasma which is much higher than normal in a suspension of shrunk and probably more rigid cells (Zade-Oppen, unpublished) (also in sickled cells: Tosteson, Carlsen and Dunham 1955). Olmstead (1960) who also neglected the interstitial volume obtained an extrapolated relative minimal volume of 0.6 for rabbit cells.

Takei obtained the smallest volume at a salt concentration of about 0.72 M NaCl, while Penati and Donati (1938) required between 0.5 and 0.7 M. In view of both the probable difficulty in defining a minimal volume and the uncertainty in the hematocrit values (where no correction is made for the trapped volume) it can only be said that the present value of 0.65 M is of the same order. However Parpart and Eggers (1950) showed that absorbance of light in a fresh red cell suspension was independent of salt concentration when this was above about 0.64 M NaCl. From this fact, and from the appearance of red cells under the microscope they concluded that the majority of cells may not alter their volume with increases in NaCl concentration above this value. This is in very good agreement with the value calculated above.

The minimal internal salt concentration in the shrunken state which on transfer to 0.155 M NaCl will cause the volume to increase to 1.6 (Ponder 1948, Hoffman et al. 1958) times the isotonic volume (V_i) (without any salt exchange) can be calculated as 1.28 M NaCl.

If, as in the experiment where the hypertonic level was 1.55 M, the cells are transferred to 0.234 M instead of 0.155 M NaCl, they should require an internal concentration of 1.93 M NaCl if osmotic equilibrium is to produce a critical volume of 1.6 V_i. This value is, however, higher than 1.55 M and can only be explained by a lower hemolytic volume (1.36 V_i) or by a false assumption in the calculation. It is for example not unlikely that the calculated concentration difference may be misleading because the activity coefficient inside and outside the cells are dissimilar.

The relationship between the concentration of the hypertonic environment and the time the cells require to be in it to give subsequently 50 per cent posthypertonic hemolysis is shown in Fig. 10.

In attempting to fit a curve to the points of Fig. 10 the working hypothesis

used to formulate an equation based on the assumption that the permeability is constant, and equal for Na and K. (As anion movements are very rapid (Tosteson 1959) it is only necessary to take into account the rate of cation transport when there is a net flow of salt.)

According to the hypothesis a cell will hemolyse in an isotonic solution if the internal concentration of permeable solutes (C_i) reaches (or just exceeds) a certain valute (C_{crit}) when the cell is in a "maximally" shrunken state.

The flux of ions into the cell, assuming an uncharged (non-ionic) cell membrane, may follow the Behn formula (Teorell 1953 p. 322). As an approximation the influence of the membrane charge can be assumed to be negligible. Furthermore, if the internal membrane potential is quite small then the Behn formula reduces to a Fick equation (Eq. 1). It thus follows that in the hypertonic environment C_i changes with time as a function of a "permeability" coefficient (P) and the concentration differences $C_m - C_i$, where C_m = concentration in medium.

The following boundary conditions are assumed. At $t = 0$ $C_i = 0.648$ at $t = \infty$ $C = C_m$. Further C_{crit} is assumed to be equal to the lowest value of C_m which will give 50 per cent posthypertonic hemolysis, i.e. 1.50 M NaCl.

$$\text{Thus} \qquad \frac{dC_i}{dt} = P(C_m - C_i) \qquad (1)$$

$$\text{and} \qquad P = \frac{RTu\ A}{\delta} \qquad (2)$$

Where RT = gas constant × absolute temperature
u = absolute mobility of cations within the membrane
A = membrane area
δ = membrane thickness

[illegible] ting (1) and then inserting the figures for C_{crit} and C at $t = 0$ gives

$$C_m = \frac{1.50 - 0.648\ e^{-P\tau}}{1 - e^{-P\tau}} \qquad (3)$$

where τ is the time during which the cells must be exposed to C_m to produce 50 per cent hemolysis.

This curve does not fit the experimental points at all well (Fig. 10). However a good fit can be obtained if it is assumed that P increases with C_m. (P values are given in Fig. 10.) It must be noted that the P values are calculated from experiments during which there is a change in the concentration difference between cell and medium and they were probably thus not quite constant in each experiment. The value of P at 1.55 M is 0.05 which is in fairly good agreement with the value calculated for the exchange rate constant for ^{22}Na for the most rapidly exchanging compartment for which the half time was 9.16 ± 1.85 min (Glaser and Wollenberger 1965). (P = 0.05 corresponds to a half time of 13.9 min).

The great increase in P as the concentration approaches 3 M, appears to be in agreement with the observations of Lovelock (1953) which led him to conclude that "the destructive action of salt solutions is complex, and manifests itself in several ways which appear to be qualitatively different" He states that between 0.8 and 3 M the cells appear to become permeable to sodium ions and presumably a new osmotic equilibrium is reached He further stated that exposure of the cells to concentrations exceeding 3.0 M should lead to their complete destruction. The process appears to be connected with the lyotropic properties of NaCl solutions"

If P increases sharply in the region of 3 M as shown in the inset of Fig 10, the ability of the cells to maintain differences between the inside and outside concentrations will probably be so greatly reduced that *colloid osmotic* hemolysis (Wilbrandt 1941) will ensue.

Concentration has been used as a measure of osmotic activity No regard has been taken of the osmotic pressure of hemoglobin solutions (Adair 1925 McConaghey and Maizels 1961) (see also Svedberg and Pedersen 1940 Rossi Fanelli, Antonini and Caputo 1961 Guidotti and Craig 1963 Benesch, Benesch and Macduff 1964) Even if equation 3 is modified (as in Fig. 10) because P is a f nction of C_{m}, it still remains a rather crude expression of the working hypothesis.

The P (C_{m}) curve of Fig 10 does, however uggest that the permeability is a monotonic function of the external NaCl concentration.

b) *Additional support for variation of "permeability" w th on centration.* An increase in red cell ghost permeability to Na and K has been found in experiments w th increasing electrolyte concentrations in the range 0 1 to 0 6 M NaCl (Passow H. Personal communication) It seems *plausible* to suppose that this increase continues also above this concentration range into the one studied in the present investigation. Passow observation would therefor appear to support the above conclusion about increasing permeabilit and thus th hypothesis that posthypertonic hemolysis is of an osmotic nature

c) *Reasons for d penden f permeabil ty upon at ation.* The cause of the increase in permeability with ncrease of electrolyt oncentration is open for discussion.

The red blood cell ma be regarded as an inner sol tion separated from the medium by a membrane possessing fixed positi e harges (.g Passow 1965) When the electrolyte concentrations re low n both sides of the membrane a positi ely charged membrane has low net tio permeability because of Donnan exclusion If the cell is now transferred to high salt concentration, an outward movement of water will occur nd the salt concentration o both sides of the membrane will then be high. This w ll reduce the cation exclusion effect of the charges and ion fl x will be increased (Teorell 1953 p 322 och 323 The Donnan exclusion is expressed by equation 8 *l.c* and he influenc on cation fluxes by equations 12 *l.c* and 16 *l*

Posthypertonic hemolysis is obtained however also when sucrose is ued to raise the tonicity (Vald neso nd H mer 1961 and has quantitatively bou the same effect as NaCl of similar osmolalit (Zade-Oppen, to be published I has

been shown whether the permeability for sucrose is also concentration dependent. However with regard to the normally very low sucrose permeability it seems plausible to suppose that the sucrose permeability must also increase if the effect observed is also to be explained as an osmotic effect as postulated for electrolytes. If a single mechanism is responsible for both posthypertonic hemolysis with electrolytes and sucrose, this cannot be due, predominantly at least, to a Donnan fixed charge effect, which would not influence, directly sucrose permeability. Thus the simplified treatment in equations 1 to 3 above will probably contain the functions necessary to express formally the dependence of permeability upon concentration. From equation 2 it is clear that it must be the membrane that becomes altered at the high solute concentrations, by changing either its geometry (thickness or diffusion area) or its structure (involving an altered cation mobility) or both. More than one mechanism may of course, exist.

Individual variations

The original aim of the experiments of Fig. 8 and 9 was to obtain an estimate of the variations between bloods from different individuals. Variations in the time curve (Fig. 9) are rather wide but as is, however evident in Fig. 10 they must be expected to be wide at the concentration used in Fig. 9. An analysis of variance showed that the spread is an actual spread from experiment to experiment and not only due to experimental errors. Even when the experiments were performed under otherwise standardised conditions, slight differences in evaporation (e.g. due to different degrees of ambient humidity or temperature) during the experiments must have been present. This will cause a change in the result which may easily be interpreted as due to individual differences. The result, however clearly demonstrates that in comparing posthypertonic hemolysis in two slightly different media at this critical concentration, the two experiments should be done simultaneously on the same blood.

Kidney

According to the counter-current hypothesis red cells are transiently exposed to a hypertonic environment during their passage through the kidney when this produces concentrated urine. According to Ullrich (1959) the tonicity change is due to an increase in NaCl and urea. Concentrations of 0.40 M NaCl and about 0.83 M urea have been measured on sections of dog kidneys. The time limits are given by the transit time for red cells passing through the papillary region of the renal medulla, i.e. up to 78 seconds (Ullrich 1959). See also Grängsjö, Ulfendahl and Wolgast (1966). It must therefore be considered whether such conditions could cause posthypertonic hemolysis. As there is normally no measurable hemolysis in the kidney red cells appear to resist the environmental changes in the kidney. If however they cannot resist the postulated concentration changes, these must, in fact, be smaller.

Lovelock found that a [illegible] up to 0.8 M and back was resisted by the cells. He did not, however include urea, which is present in the medulla at high concentrations. Urea at a concentration [illegible] per cent in 0.9 per cent NaCl gives hemolysis

after return to 0.9 per cent NaCl with dog and rabbit erythrocytes (Pinter and Zilversmit 1960). Söderström (1944) stated that urea dissolved in isotonic NaCl caused hemolysis at approximately the same osmotic pressure as corresponding hypertonic NaCl solutions.

Red cells were exposed to solutions each containing both NaCl, in concentrations greater than postulated for the renal medulla of the dog and urea (0.667 and 0.8 M). Even after exposure for periods longer than the probable time, during which the cells pass through the hypertonic medullary region, hemolysis was insignificant. With regard to the sizes of human and dog kidneys and their ability to produce concentrated urine, it seems likely that also other conditions are similar for these species.

It thus appears that the red cells can tolerate the renal conditions postulated in the counter-current hypothesis.

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centration depolarizes the cell membranes in guinea-pig taenia coli (e g Holman 1958) and also that the same smooth muscle cells are hyperpolarized by decreased external sodium concentration and depolarized by reduced chloride concentration (Kuriyama 1963)

However several authors have reported ionic effects on the membrane potential of smooth muscle which do not correspond to those predicted by merely inserting the actual ion concentration values in the constant field equation. As a rule these unexpected responses have occurred on sudden changes in ion concentrations and they have mostly been transitory For instance, Holman (1958) and Kuriyama (1963) reported that the immediate effect of potassium-free solution on the taenia coli was depolarization and increased activity but hyperpolarization occurred on prolonged exposure. Studies on spontaneously active preparations from guinea pig uterus and frog stomach have shown that an increase in the external potassium ion concentration inhibits transiently the activity of these muscles (Klingenberg 1962) In potassium-free solution the isolated rat uterus developed a "contracture upon which phasic contractions were superimposed (Kuschinsky Lüllmann and Mosler 1960) Rapid relaxation and transitory cessation of the spontaneous contractions were seen when the potassium concentration was normalized again.

Also certain adjustments in the activity of cardiac and smooth muscle occurring in vivo in response to ionic changes are difficult to explain on the basis of current concepts Thus moderate increases in arterial potassium ion concentration cause peripheral vasodilatation (e g Emanuel, Scott and Haddy 1959 Kjellmer 1965) and under certain circumstances temporary cardiac arrest (Surawicz and Gettes 1963).

It was demonstrated in an earlier report from this laboratory (Axelsson et al 1967) that the smooth muscle of the rat portal vein shows a transitory depolarization with increased spike activity on reduction of the external potassium ion concentration, and that marked inhibition of activity occurs on restoration of $[K^+]_o$. In the course of a series of experiments in which the effects of lowered and restored chloride and sodium concentrations were investigated, we noticed some remarkable similarities between the responses to these ionic changes and the responses obtained in the previous study by changing potassium concentration. It seems as if many of these responses cannot be accounted for by merely adjusting the concentration values in the constant field equation.

The aim of the present study was to characterize in some detail the reactions of the smooth muscle of the portal vein to decreased and restored concentrations of potassium chloride and sodium ions.

Methods

This report is based on 48 experiments in which the mechanical activity of the rat portal vein was recorded, and 8 experiments in which the simultaneous electrical activity was registered as well. The sucrose gap technique was used for studying electrical activity

The preparation procedure and the recording techniques have been described in greater detail in a previous article (Axelsson et al 1967 Female rats, weighing 150—200 g were used. In the experiments where only mechanical activity was recorded, 0.5—1 cm long section of the portal

TABLE I. Composition of solutions in mmoles/l

Solution	Na^+	K^+	Cl^-	Ethane-sulph.	Tris	HCO_3^-	$H_2PO_4^-$	Mg^{++}	Ca^{++}	Glucose	Sucrose
Normal	137	5.92	134	—	—	15.5	1 19	1 19	2.49	11.5	—
K free	143	—	134	—	—	15.5	1 19	1 19	2 49	11.5	—
Cl^- low	137	5.92	12.0	122	—	15.5	1 19	1 19	2.49	11.5	—
Na low	15.0	5.92	120	—	136	15.5	1 19	1 19	2.49	11.5	—
K free and Cl^- low	143	—	12.0	122	—	15.5	1 19	1 19	2.49	11.5	—
Na low and Cl^- low	15.0	5.92	12 0	—	—	15.5	1 19	1 49	2 49	11.5	44

vein was mounted in mantled organ bath and connected to an isometric tension transducer (Grass FT 03) under passive tension of about 400 dyn. The tension development of the preparation was recorded on Grass polygraph.

A somewhat longer piece of the ein was prepared for simultaneous recording of mechanical and electrical activity. In these experiments the hepatic end of the ein was connected to force displacement transducer and superfused with normal Krebs solution or test solutions. The mesenteric end was depolarized by a Krebs solution in which NaCl had been replaced by KCl. The central part of the preparation was superfused with isotonic sucrose solution. The potential differences between the polarized (hepatic) and depolarized (mesenteric) end of the ein and the tension development were monitored on double beam oscilloscope and recorded on moving film.

The normal Krebs solution and five special solutions (T ble I) were used to obtain graded decreases in the concentrations of K N and Cl^- Potassium ions were substituted with sodium ions, chloride ions with ethanesulphonate or sucrose while sodium ions were replaced by Tris or sucrose. The slight increase in $[Na]_o$ bove normal in the potassium free medium is evidently without importance for the activity of the muscle. The sodium low solution was prepared by titrating 0 100 N HCl with Tris base to pH of 7.30 37° C. The other components contained in the normal medium were added and the solution was bubbled before the last pH adjustment. It should be noted that the chloride concentration is 14 meq/l lower in the sodium-low han in the normal medium. Normal $[Cl^-]_o$ could have been obtained by using 0.122N HCl but the amount of Tris base required for titration to normal pH would then ha made the solution hyperosmotic. Due to the sensitivity of the vascular smooth muscle to changes in external osmolarity (Johansson and Jonsson 1967) we ha preferred to use the slightly chloride deficient but isoosmotic solution.

Apart from the possible reductions in the concentrations of single ions or of pairs of ions, combinations of the solutions given in T ble I allow reciprocal ariations in $[Cl^-]_o$ on the one hand and $[K]_o$ or $[Na]_o$ on the other. All solutions were bubbled with gas mixture of 96 O and 4 CO_2 giving pH of 7.30. The bath temperature was kept 37° C. A motordriven syringe which delivered constant volume of pre-warmed fluid to the bath per minut was used for experiments, in which slow changes in the ion concentrations were desired.

The chemical compounds used to substitut for chloride and sodium ions were sodium ethanesulphonate (K&K laboratories Inc.) and tris-(hydroxymethyl)-amino-methane (Trizma Base Sigma Chemical Company) respectively

Results

The spontaneous activity of the isolated rat portal em in normal Krebs solution onsists of phasic contractions occurring at fairly regular intervals These ntrac tions are strictly correlated with action potentials which appear in bursts f ariable duration (Axelsson *et al.* 1967)

The effects on this spontaneous activ ty of comparabl decreases in the concentra

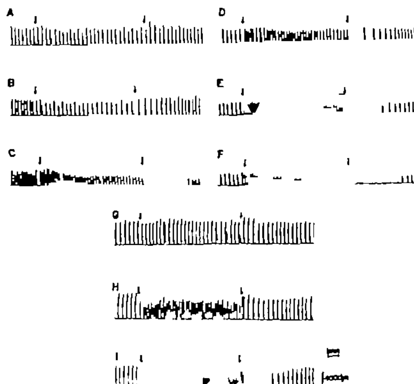


Fig. 1 Effects on the spontaneous activity of the rat portal vein of comparable decreases in potassium, chloride and sodium ion concentrations during the periods indicated. The following changes in ionic composition of external fluid were done, substituting N for K, $C_6H_5SO_3^-$ for Cl^- and Tris for Na
A. $[K]_o$ 4.2 meq/l B. $[K]_o$ 2.4 meq/l C. $[K]_o$ 0.6 meq/l. D $[Cl]_o$ 94 meq/l E. $[Cl^-]_o$ 51 meq/l F $[Cl^-]_o$ 12 meq/l G $[N]_o$ 96 meq/l. H. $[N]_o$ 55 meq/l. I. $[N]_o$ 15 meq/l

tions of potassium, chloride and sodium ions are illustrated in the mechanical recordings of Fig 1 There are some conspicuous similarities between the responses obtained at the largest of these changes in ionic environment. Reducing the concentration of any of the three different ions down to 10 per cent of the normal thus led to a marked initial excitation whereas the first minutes of the recovery period in normal solution were characterized by total inhibition of activity (Fig 1 C F I). These common transient reactions, obtained on large reductions in ion concentrations and on readministration of normal solution, will be subjected to further anal n the experiments reported below It may be appropriate however first to consider briefly the effects of the more moderate changes in environment which are hown in Fig 1

Reducing the potassium ion concentration to 70 and 40 per cent of its normal value in Fig 1A and B respectively leads to some decrease in contraction frequency during the posure periods. Transient reactions of the kind seen at the beginning and the end of the exposure period in Fig 1C are not obvious in A and B. The slowing of the ontra tion frequency produced by moderately decreased

$[K^+]_o$ is associated with hyperpolarization and inhibition of spike discharge as shown in a previous study (Axelsson *et al.* 1967) and confirmed in experiments of the present series including registration of electrical activity.

When chloride ions were substituted with ethanesulphonate down to 70 and 40 per cent of the concentration in normal solution (Fig. 1D and E, respectively) there was an excitatory pattern of response characterized by an increased frequency of contractions which prevailed throughout the exposure periods. Contraction frequency is relatively greater in the initial phase and inhibition of activity is seen on return to normal solution. The transient reactions associated with the sudden changes in ionic environment are thus apparent also at more moderate variations in $[Cl^-]_o$ and they are quantitatively dependent on the magnitude of the ionic change. Sucrose-gap experiments showed a pronounced depolarization in chloride-low solutions and the increased contraction frequency reflected a decrease in the intervals between the bursts of action potentials. The disproportionately large depolarization obtained in the sucrose-gap recording by reduction in the chloride content of the medium have been described previously and interpreted as an artifact due to liquid junction potentials (Fariduddin and Armstrong 1965).

Substituting 30 per cent of the sodium ions of the normal solution with Tris has little influence on muscle activity (Fig. 1 G) but a 60 per cent exchange leads to a sustained increase in frequency (H). Transient reactions to the ionic shifts are not obvious in these recordings. The initial transient excitation obtained by substituting 90 per cent of the sodium ions with Tris in Fig. 1 I does not distinguish itself very clearly from the sustained increase in frequency but the period of quiescence on return to normal solution is unequivocal.

The purpose of the following experiments will be to characterize in some detail the transient responses which are common to the three ion species as illustrated in Fig. 1 C, F and I.

Changes in $[K^+]_o$

As indicated in Fig. 1 no excitation appeared as a rule on decreases in potassium concentration from the normal to a level of approximately 2 meq/l or above. The muscle became excited more easily on decreases below this level. Furthermore, the transient excitation on lowering of the potassium content disappeared if the ionic change was done slowly or in small steps.

This is illustrated in the experiment of Fig. 2 where the effects of a sudden decrease in $[K^+]_o$ to 0.6 meq/l (A) were compared with those of a slow continuous decrease from 2.0 to 0.6 meq/l over approximately 20 min after a rapid reduction from 5.9 to 2.0 meq/l (B). The marked excitation on the sudden decrease to 0.6 meq/l and the lack of excitation when the potassium concentration was lowered slowly should be noted. A decrease in the potassium concentration from 2.0 to 0.6 meq/l over approximately 10 min instead of 20 resulted in an clearcut increase in the contraction frequency (not shown in the figure).

The duration of the inhibition of portal vein activity seen on restoration of the

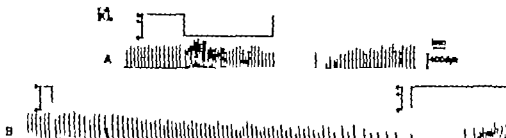


Fig. 2. Responses of portal vein t brupt and gradual reductions in $[K]_o$.
A. Abrupt reduction in $[K]_o$ from 5.9 to 0.6 meq/l
B. Abrupt reduction in $[K]_o$ from 5.9 to 2.0 meq/l followed by a slow continuous reduction to 0.6 meq/l.

potassium ion concentration turned out to be dependant on the level to which $[K]_o$ had been reduced, on the duration of the exposure to potassium low medium, on the level to which the potassium ion concentration was increased and on the rate at which this increase was done. The importance of the magnitude of the increase is shown in Fig. 3. In each recording of this figure the potassium ion concentration was suddenly reduced from normal to 0.6 meq/l and maintained at this level for approximately 5 min. Increasing potassium concentration to 1.2 meq/l only (Fig. 3 A) caused a slowing of contraction frequency but the longlasting cessation of the phasic activity did not occur until $[K]_o$ was abruptly increased to the normal level. A brief period of quiescence is seen in Fig. 3 B when $[K]_o$ was changed from 0.6 to 2.0 meq/l and later another phase of complete inhibition appeared on return to normal solution. In Fig. 3 C muscle activity stopped for about 1.5 min on increasing $[K]_o$ from 0.6 to 4.0 meq/l but was relatively little influenced by the further increase to 5.9 meq/l.

The results illustrated in Fig. 3 show that the magnitude of the increase in $[K]_o$ ... es the duration of the complete inhibition and also that the muscle is ... sensitive to this influence of potassium at a concentration level below 4 meq/l.

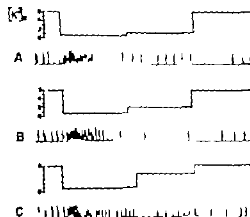


Fig. 3. Responses of portal vein to graded increases in $[K]_o$ from 0.6 meq/l after 5 min in this potassium-low medium.
A. $[K]_o$ increased to 1.2 meq/l in the first step and then, after 5 min to 5.9 meq/l
B. $[K]_o$ increased to 2.0 meq/l in the first step and then, after 5 min to 5.9 meq/l.
C. $[K]_o$ increased to 4.0 meq/l in the first step and then, after 5 min to 5.9 meq/l

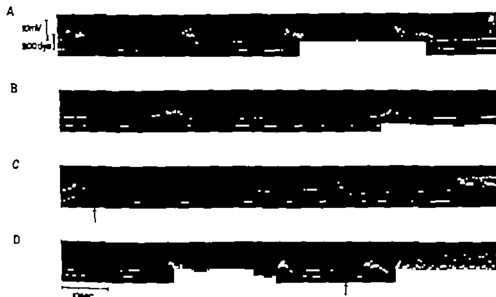


Fig. 4. Spontaneous electrical (upper tracings) and mechanical (lower tracings) activity of rat portal vein and its responses to variations in $[K^+]_o$.
A. Normal solution.
B. After 10 min in solution with 0.6 meq K^+/l.
C. $[K^+]_o$ increased from 0.6 to 36 meq/l at arrow.
D. $[K^+]_o$ increased from 5.9 to 36 meq/l at arrow.

It may be pointed out in this connection, however, that sudden increases in $[K^+]_o$ also to supranormal levels after a period in low potassium lead to cessation of contractile activity for variable periods of time. The effects of a sudden increase in potassium concentration from 0.6 meq/l to supranormal levels are illustrated in Fig. 4. In this experiment electrical and mechanical activity were recorded together. The activity in normal Krebs solution is shown in Fig. 4 A and the activity after 10 min in 0.6 meq K^+/l in 4 B. The initial excitation had, at that time, elapsed and contraction frequency and amplitude had decreased compared to Fig. 4 A. At the arrow in Fig. 4 C the potassium concentration was increased from 0.6 to 36 meq/l. A state of continuous spike firing and tetanic tension development, typical for the muscle in this high potassium ion concentration (*cf.* Axelsson *et al.* 1967) was ultimately obtained but the onset of the increased activity was considerably delayed. This excitation manifested itself almost instantaneously on shifting from the normal solution containing 5.9 meq K^+/l to the solution with 36 meq/l (4D).

The importance of the rate of the increase in $[K^+]_o$ is shown in Fig. 5. An abrupt increase in $[K^+]_o$ from 1.0 meq/l to 5.9 meq/l after 4 min in the potassium low medium brought about an approximately 80 sec long cessation of the spontaneous activity (5 A). When the potassium concentration was gradually raised at a re

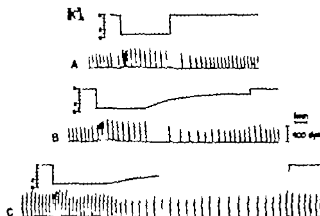


Fig 5. Responses of portal vein to abrupt and gradual increases of $[K^+]_o$ after 4 min in a potassium-low solution.
A. Abrupt increase in $[K^+]_o$ from 1 0 to 5.9 meq/l
B. $[K^+]_o$ gradually increased from 1 0 to 4.0 meq/l during 9 min period.
C. $[K^+]_o$ gradually increased from 1 0 to 4 0 meq/l during 14 min period.

high rate a considerably longer period of quiescence occurred (5 B) An increase in $[K^+]_o$ from 1 0 meq/l at a much slower rate caused a slowing of contraction frequency (5 C) but not the complete cessation of spontaneous activity seen in A and B.

Changes in $[Cl^-]_o$

Fig 6 illustrates some further aspects of the effects of decreased Cl concentration. The pronounced initial excitation, seen on a sudden reduction of the Cl concentration to 45 meq/l (Fig 6 A) was absent when the Cl concentration was slowly reduced to the same level (B) The 2 min long silence seen on the sudden normalization of the chloride concentration after 4 min in 45 meq Cl /l (Fig 6 C) did not occur in D where $[Cl^-]$ was increased slowly It is evident from Fig 6 that the transient responses to reduction in $[Cl^-]_o$ and to readministration of solution with normal Cl content are dependent on the abruptness of the ionic shifts

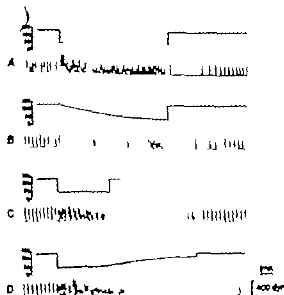


Fig 6 Responses of portal vein to abrupt and gradual decreases and increases of $[Cl^-]_o$.
A. Abrupt decrease of $[Cl^-]_o$ from 134 to 45 meq/l and abrupt normalization of $[Cl^-]_o$ after 9 min.
B. Slow gradual decrease of $[Cl^-]_o$ from 134 to 45 meq/l during 9 min period and after that, rapid normalization of $[Cl^-]_o$.
C. Abrupt decrease of $[Cl^-]_o$ from 134 to 45 meq/l and abrupt normalization of $[Cl^-]_o$ after 4 min
D. Abrupt decrease of $[Cl^-]_o$ from 134 to 45 meq/l and after 4 min, slow continuous increase during 14 min period.

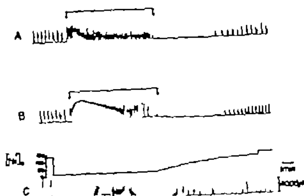


Fig. 7 Responses of portal vein to variations in $[Cl^-]_o$ and $[Na^+]_o$ respectively and to combined changes in the sodium and chloride concentrations.
A $[Cl^-]_o$ reduced from 134 to 12 meq/l NaCl substituted by sodium ethanesulphonate.
B $[Na^+]_o$ and $[Cl^-]_o$ reduced from normal values to 15 and 12 meq/l, respectively; NaCl substituted with sucrose
C. $[Na^+]_o$ reduced from 137 to 15 meq/l substituted with Tris and after 8 min, slowly increased to 110 meq/l during 10 min period.

Changes in $[Na^+]_o$

The substitution of Na with Tris resulted, as described above, in a sustained excitation which was somewhat more intense in the beginning of the exposure period. It is essential to ascertain whether this response is due to the decrease in $[Na^+]_o$ itself or whether it is a non-specific effect of the Tris ions or of the small amounts of undissociated Tris which are necessarily present in this solution. Other non-permeant Na substitutes should be tested to see whether these would change muscle activity in a similar fashion as Tris. Choline chloride which is widely used as a substitute for NaCl appears to be less suitable for the present experiments due to the strong cholinergic responses that it produces in the portal vein. It was decided, therefore, to examine the importance of decreased $[Na^+]_o$ by comparing the effects of NaCl substitution by sucrose and by sodium ethanesulphonate, respectively. The experiment illustrated in Fig. 7 A and B was performed with this intention. In Fig. 7 A the chloride concentration was reduced to 12 meq/l, by substituting NaCl with sodium ethanesulphonate and in Fig. 7 B, the same reduction in Cl concentration was done simultaneously with a decrease in sodium concentration to 15 meq/l, both ions being substituted for with equiosmolar amounts of sucrose. There was a much more pronounced initial excitation when the sodium concentration was reduced as well. The sustained excitation obtained with Tris substitution in Fig. 1 H and I did not occur with sucrose (Fig. 7 B).

Fig. 7 C shows that it is possible to increase the sodium concentration from 15 meq/l to the normal value of 137 without producing the conspicuous inhibition of activity seen in Fig. 1 I by doing it slowly during an approximately 6 min period. A gradual increase of the sodium concentration at a higher rate could result in an abolishment of spontaneous contractions that lasted even longer than when sodium concentration was restored abruptly (not shown in the figure).

Reciprocal variations in anion and cation concentrations

It is evident from the results reported before that the inhibition seen on restoration of potassium, chloride and sodium ion concentrations respectively could be avoided

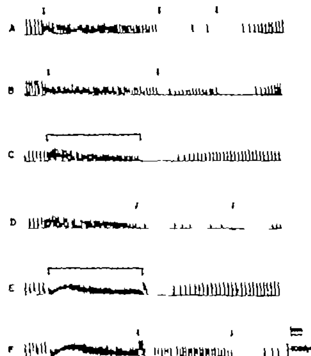


Fig. 8. Responses of the portal vein to reciprocal variations in the concentrations of anions and cations. Normal Krebs solution before and after the periods indicated.
A. Reduction in $[K^+]_o$ to 0.6 meq/l (first period indicated) and partial restoration to 2.0 meq/l (second period). To be compared with
B. where the reduction in $[K^+]_o$ to 0.6 meq/l (first period) is followed by partial restoration of $[K^+]_o$ to 2.0 meq/l and simultaneous decrease of $[Cl^-]_o$ to 45 meq/l.
C. Reduction in $[Cl^-]_o$ to 45 meq/l. To be compared with
D. where the reduction in $[Cl^-]_o$ to 45 meq/l (first period) is followed by restoration of $[Cl^-]_o$ to normal and simultaneous decrease of $[K^+]_o$ to 0.6 meq/l (second period).
E. Reduction in $[Na^+]_o$ to 15 meq/l. To be compared with
F. where the reduction in $[Na^+]_o$ to 15 meq/l (first period) is followed by restoration of $[Na^+]_o$ to normal and simultaneous decrease of $[Cl^-]_o$ to 45 meq/l (second period).

to a great extent by making the ionic changes sufficiently slow. This inhibition could also be eliminated by combining the abrupt restoration of the cation concentrations with a simultaneous abrupt decrease in $[Cl^-]_o$ or *vice versa*. This is illustrated in Fig. 8.

In the recording of Fig. 8 A the external potassium ion concentration was varied while $[Cl^-]_o$ was kept constant at the normal level throughout. Shifting from solution with 0.6 meq K^+/l (first exposure period indicated in Fig. 8 A) to one with 2.0 meq/l (second exposure period indicated) abolished contractions for approximately 3 min and another period of quiescence occurred on the final return to normal solution. This recording should be compared with Fig. 8 B in which $[Cl^-]_o$ was reduced to about one third of its normal at the same time as $[K^+]_o$ was approximately tripled (second arrow in Fig. 8 B). It is evident that the inhibition observed during the second exposure period of Fig. 8 A is almost completely abolished in the corresponding period of B where the potassium increase was combined with chloride reduction. Restitution of $[Cl^-]_o$ and $[K^+]_o$ to normal in Fig. 8 B (third arrow) is associated with a long lasting inhibition as expected.

The analogous effect of a simultaneous decrease in $[K^+]_o$ on the inhibition produced by restoring $[Cl^-]_o$ from low to normal level is illustrated in Fig. 8 C and D. The 4 min period of quiescence obtained in Fig. 8 C by increasing $[Cl^-]_o$ from 45 meq/l to 134 meq/l is thus absent in the second exposure period of Fig. 8 D where the return to normal chloride was combined with a reduction of $[K^+]_o$ to 0.6 meq/l.

It is notable that a proportionately greater decrease in potassium ion concentration was necessary to counteract the effect of a certain increase in $[Cl^-]_o$.

The last two recordings of Fig. 8 show how the inhibition following a period of exposure to Na-low environment (Fig. 8 E) can be abolished by combining the return to normal $[Na^+]_o$ with a simultaneous decrease in $[Cl^-]_o$ (second period indicated in Fig. 8 F). Chloride concentration was reduced to about one third of its normal when sodium concentration was increased approximately nine-fold in this case.

Discussion

This paper describes influences on spontaneous activity in vascular smooth muscle of changes in the ionic composition of the extracellular fluid. These ion variations should exert their effects on muscle activity primarily by altering the electrical characteristics of the cell membranes. We have chosen to illustrate the results of this study mainly by oscillograph recordings of mechanical activity due to the better surveyability of these records compared to the photographic tracings of mechanical and electrical events obtained in the sucrose-gap experiments. It is clear, however, from the latter type of experiments that the contractile activity in this muscle is well correlated with the electrical spike discharge (Fig. 4 above) which justifies a discussion of electrical phenomena on the basis of the mechanical illustrations. The sucrose-gap experiments further indicate that the changes in burst activity are mediated *via* variations in the level of the membrane potential although we cannot express this relationship quantitatively.

The reactions seen immediately after the rapid changes in the ionic concentrations are the main topic of the present paper but the more sustained changes in muscle activity observed during the later phases of the exposure periods will first be briefly discussed.

Moderate sustained reductions in $[K^+]_o$ lead to hyperpolarization and inhibition of spike activity in the smooth muscle of the portal vein as demonstrated in a previous report (Axelsson *et al*, 1967). This response is in agreement with that expected on the basis of the constant field equation (see above). At more pronounced decreases in $[K^+]_o$ tension development is impaired and finally both electrical and mechanical activity are completely abolished.

The sustained effects of decreased $[Cl^-]_o$ are also in accordance with the constant field equation. Depolarization and increased frequency of bursts were thus seen and this activity persisted for at least 30 min in the chloride-low medium.

The maintained excitatory pattern of activity obtained at Na substitution with Tris cannot be directly reconciled with the Goldman equation, according to which the effect of reduced $[Na^+]_o$ should be hyperpolarization provided that ion permeabilities remained constant. It is possible that the undissociated Tris contained in the isosmotic Na-low solution permeates the cell membranes to cause osmotic swelling and excitation (*cf.* Johansson and Jonsson 1967) and that this mechanism contributes to the sustained response in Fig. 1 H and I. The comparison between NaCl solut

tion with sodium ethanesulphonate and with sucrose, respectively in Fig 7 A and B indicates that the long-term effect of reduced [Na] on the portal vein is indeed an inhibitory one as seen from the lower contraction frequency in the later part of the exposure period in B compared to A.

The sustained patterns of muscle activity observed in different ionic environments are thus in agreement with the changes in membrane potential that could be predicted by considering the role of the ion concentrations in the constant field equation. Several of the immediate responses associated with rapid changes in the ionic environment cannot however be explained on the basis of the Goldman formula by simply adjusting the concentration values. This is most obvious when the potassium concentration is reduced markedly and when it is restored again to normal or supranormal levels. The excitatory response of the portal vein on reduction of $[K^+]_o$ and the complete cessation of spontaneous activity on return to normal solution were described in our previous report (Axelsson *et al* 1967) and similar reactions have been observed in other smooth muscles as mentioned above (Kuschinsky Lüllmann and Mosler 1960 Klingenberg 1962, Kuriyama 1963). The possibility that membrane permeability to potassium is reduced in the portal vein at low [K] was considered in our previous study (Axelsson *et al* 1967) and the immediate responses of the portal vein to variations in $[K^+]_o$ might then be explainable on the basis of the constant field equation by appropriate adjustments of the relative permeability factors (α and β above)

The present investigation has shown that the immediate responses of the portal vein to large reductions in $[K]_o$, $[Cl]_o$ and [Na] and to readministration of normal solution are remarkably similar for the three ion species. The excitation seen on reduction of the ionic concentrations and the inhibition obtained at normalization may therefore reflect some common mechanism in which these ions are all involved. One possibility is of course, that the membrane effects of the variations in ion concentrations are modified by changes in relative permeabilities which act in the same direction for any large reduction in the external concentration of the three ions and for the readministration of normal medium, respectively. It may be possible to reconcile the present observations with the constant field equation by assuming that such adjustments of membrane permeability occur on sudden changes in the ionic environment.

On the other hand, Kuschinsky *et al* (1960) suggested that the transient reactions of rat uterus to sudden reduction and restoration of $[K]_o$ might be due to transmembrane fluxes of potassium ions that temporarily would overcome the influence of the actual potassium concentration gradient on the membrane potential. With this in mind, attempts were made in the present study to eliminate the immediate portal vein responses to changes in $[K]_o$ and [Cl] by altering these ion concentrations in such a way that their product remained constant (*cf* Klingenberg 1962). This procedure would prevent net transfer of the ions across the cell membranes if K and Cl were distributed according to a Donnan equilibrium. It was found that the transient reactions of the muscle could indeed be almost completely abolished by

reciprocal variations in anion and cation concentrations (Fig 8) but the necessary adjustments of the concentrations did not correspond to a constant product of $[K^+]_o$ and $[Cl^-]_o$. Also the immediate effect of increased $[Na^+]_o$ was cancelled by a simultaneous decrease in $[Cl^-]_o$. Transmembrane transfer of ions due to disturbance of a Donnan balance in which Cl, K and Na are all involved may possibly account for the transient portal vein responses which are common to the three ions In such transmembrane ion fluxes cations and anions would be involved in equal proportion for the maintenance of electroneutrality Differences between the ions in their ability to pass through the membrane could, however lead to some separation of the oppositely charged ions so that a diffusion potential is established over the membrane This diffusion potential could in its turn influence the level of the membrane potential and modify the effects of the concentration and permeability factors expressed in the Goldman equation. However no definite conclusions as to the nature of these transient responses can be drawn, particularly since the ionic distribution is not known for this vascular smooth muscle.

Experiments on taenia coli by Casteels and Kuriyama (1966) indicated that a Donnan situation does not prevail in smooth muscle. They found that the equilibrium potential for Cl calculated from determinations of $[Cl^-]_i$ and $[Cl^-]_o$ did not correspond to the "resting" potential of the taenia coli cells and suggested therefore that Cl is not passively distributed in these cells It seems, however that it would have been more appropriate to compare E_{Cl^-} with the effective average membrane potential of the spontaneously active taenia coli as done for heart muscle by Woodbury (1962, p. 242—243)

The present in vitro results may at least as far as the potassium ion is concerned, have some implications for vascular smooth muscle activity under in situ conditions since variations in the plasma concentration of this ion induce certain circulatory responses which are not readily explained on the basis of current concepts Vasodilatation was thus induced in dog forelimb and cat hind leg, respectively by close arterial infusions of isotonic solutions of potassium salts which increased venous plasma concentration from between 3 and 4 meq/l to about 8 meq/l Emanuel, Scott and Haddy 1959 Kjellmer 1965) Corresponding increases in interstitial potassium ion concentration occurred in skeletal muscle during work and a role of the potassium ion as a vasodilator in exercise hyperemia was postulated therefore (Kjellmer 1965) Changes in the external potassium ion concentration over the same range in the present experiments on isolated rat portal vein resulted in a clear-cut, transient inhibition of spontaneous activity

The cellular mechanisms behind the potassium vasodilatation have not been adequately explained It has been ascribed to an increased membrane permeability to potassium produced by the increase in the external concentration of this ion Johansson and Bohr 1966) This change in permeability could result in hyperpolarization and thereby cause inhibition of vascular smooth muscle activity Transmembrane fluxes of ions as discussed above might offer an alternative explanation for the potassium vasodilatation.

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The Metabolism of Human Spermatozoa in the Presence of Prostaglandin E

By

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Abstract

Eliasson, R., R. N Murdoch and I G White. *Th metabolism f human spermatozoa the presen of prostaglandin E* Acta physiol. scand. 73 379—382.

The oxygen uptake and metabolism of human spermatozoa ha been measured using concentrated suspensions of cells incubated with radioactiv fructose in small Warburg flasks. The oxygen uptake was about 14 µl per 10^9 sperm pe hr nd represented true respirator activity since radioacti carbon dioxide was produced as well as lactic acid.

The presence of 100 µg of prostaglandin E pe ml had no effect on oxygen ptake, the amount of fructose metabolised or the amount of lactic acid accumulating

The chemical structure of the prostaglandins has recently been elucidated (Bergström *et al.* 1962, 1963 Samuelsson 1963 Hamberg and Sam elsson 1966) but the physiological function of these unusual fatty acids in human semen is still not known with any certainty All the compounds affect the contraction of smooth muscle and it has been suggested they might play some part n the voiding of the male accessory secretions (Euler 1936) or facilitate the passage of permatozoa up the uterine cavity (Eliasson 1959) One of the main prostaglandins in human semen is PGE The possibility that this compound may influenc the metabolism of human spermatozoa is examined in this paper

M thod

Semen was supplied by 14 normal students and the time from collection to the start of the experiments was alway less than 2 hrs Ejaculates ere pooled and the spermatozoa washed by diluting the semen with 3 olumes of diluent and centrifuging for 10 min at 400×*g* The sedimented spermatozoa were washed again and diluted to the appropriate olume with the diluent used for washing. The diluen was Ca-f ee Krebs Ringer phosphate buffer (Lardy et al. 1939 containing crystalline penicillin G and streptomycin sulphate 0 5 mg of each/ml diluent)

Washed spermatozoal suspensions (0 5 ml) ere incubated 37 C with 0.5 ml of labelled fructose diluent in 6 ml Warburg flasks containing CO_2-free 20 % w/v) KOH in the centre

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Effect of Hyperbaric Oxygenation on Wound Healing and Experimental Granuloma

By

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Increased O_2-moiety in the breathing atmosphere (35—70 % v/v at 1 atm. abs. pressure) accelerated the wound healing significantly (Kulonen *et al.* 1967). We wished to extend the work to hyperbaric oxygenation, especially because the earlier reports in the literature are conflicting. Lundgren and Sandberg (1964) found that the tensile strength of surgical wounds in rats decreased when they were kept intermittently at 2 atm. of O_2, but after an acute haemorrhage hyperbaric oxygen had a stimulatory effect on the healing (Lundgren and Sandberg 1966). A beneficial influence in dogs was observed also by Beckham and Hitchcock (1964) on ischaemic wounds by Illingworth *et al.* (1961) and on skin flaps by Perrins (1966).

Viscose cellulose-sponges (four pieces, 20 × 10 × 10 mm each) were implanted subcutaneously in the backs of adult male Sprague-Dawley rats (about 200 g) which were treated after the first postoperative night for 3 or 9 days intermittently for × 2 hrs (the interval 2 hrs) daily in pure 2-atm. oxygen (in hyperbaric animal chamber by Vickers Research Establishments, Sunninghill, Berks, England. Water and food were available *ad libitum*. The flow of oxygen was 1 l/min, and the highest CO_2-content in the breathing atmosphere remained under 0.5 % v/v. The temperature in the chamber increased by 2° C and the relative humidity decreased from about 50 % to 35 % during the 2-hr exposure. No rats died during the experiments, their weight increased normally and no signs of the oxygen toxicity (e.g. convulsions) were observed.

The tensile strength in the healing skin wounds (g/cm) and in the granulomas (g/cm²) was determined as described by Viljanto (1964). Each sample in the chemical analyses consisted of four granulomas. Hydroxyproline was determined according to Woessner (1961), nitrogen by micro-Kjeldahl digestion followed by nesslerization and haemoglobin as cyanmethaemoglobin.

From Table I it is evident that the tensile strength is significantly lower on O_2-treated groups both after 4 and 10 days. The content of hydroxyproline of the granulomas changed accordingly. The decrease of the haemoglobin concentration is due either to a diminished vascularity or to a vasoconstriction caused by hyperbaric oxygen. A similar statistically non-significant reduction was observed in the contents of total nitrogen, and also of DNA and RNA, not given in detail.

Our results are in good agreement with those of Lundgren and Sandberg (1964). At 2-atm. O the optimal concentration of oxygen in the brea

TABLE I Effect of hyperbaric oxygenation on the tensile strength of skin wound and an experimental granuloma.

Variable	4-day study		10-day study		P
	Control	Experiment	Control	Experiment	
Tensile strength					
Skin wound (g/cm)	112±6 (19)	91±4 (19)	864±22 (16)	659±11 (16)	<0.005
Granuloma (g/cm)	55±3 (19)	46±2 (20)	357±17 (19)	293±15 (20)	<0.005
Chemical composition of granuloma (per 2 cm²)					
Hydroxyproline					
(μg)	149±11 (6)	124±11 (6)	1769±163 (4)	1487±83 (6)	NS
Nitrogen (mg)	6.3±1.0 (6)	5.9±0.7 (6)	9.6±1.2 (5)	9.0±1.2 (5)	NS
Haemoglobin	2.48±0.21 (6)	1.60±0.20 (6)	3.04±0.26 (5)	2.83±0.38 (5)	NS

The number of the rats is given in the parentheses and the standard error of the mean is indicated. The statistical significance was tested by analysis of variance.

atmosphere is passed. In the local treatment or in certain difficult conditions, e.g. in ischaemia, a hyperbaric pressure may be required for a sufficient oxygenation in the healing tissue.

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From the Institute of Physiology, University of Bergen, Norway

Temperature Effects on the Viscosity of Blood and the Aorta Distension from a Hibernator Erinaceus Europaeus L.

By

ARNE KIRKEBÖ

Received 17 November 1967

Abstract

KIRKEBÖ, A. *Temperature effects on the viscosity of blood and the aorta distension from a hibernator Erinaceus europaeus L.* Acta physiol. scand. 1968. 73. 385—393.

Hedgehog blood at all temperatures showed higher apparent viscosity than most other mammals and exhibited pseudoplastic characters at low rates of shear. In contrast, serum never showed shear rate dependence. Red cells in serum or Ringer solution had less shear rate dependence than did blood corpuscles suspended in plasma. The apparent viscosity of a sample doubled when the temperature fell from 34° to 6° C. The change in viscosity found in different blood samples withdrawn at 6°, 20° and 34° C during arousal from hibernation, was greater than the reduction found in one sample over the same temperature range. The cross-section of the aorta increased with falling temperatures from 34° to 6° C, but the elastic properties of the vessel remained relatively unchanged. A deviation from Hooke's law is evident, but it was not great within the normal homeostatic pressure range. Distension with increasing pressure was a little larger for vessels from non-hibernating than for hibernating animals. The hindrance of the aorta in the hibernating state was increased by a factor of 1.9 compared to the value of the non-hibernating state. If there hypothetically was no distension with falling temperature the increase in hindrance would be about 2.5.

Relative viscosities of blood from one hibernator *Citellus undulatus* have been determined only once by Hock (1964) with a Hess viscometer. He found increasing blood viscosity relative to water with decreasing temperatures.

Merrill *et al.* (1963 b) have in the last few years worked extensively on human blood viscosity at low rates of shear. They found that the relative viscosity of blood to water is independent of temperature at all rates of shear above 1 sec. *In vivo* determinations have been made by Wittaker and Winton (1933). The apparent blood viscosity in the hindlimb of dogs were only half the viscosity found in the glass viscometer.

The physiology and distensibility of the aorta wall have been thoroughly reviewed by Remington (1963) and by Bader (1963). The temperature effects on circulation are not treated. The uer (1963) gives however a general review on the subject. He says it is possible that one causative factor of cold vasodilatation is the direct effect of cold on the vessels themselves.

The work by Folkow et al. (1963) should also be mentioned. Different mechanisms are discussed that may explain the initial decrease of blood flow and subsequent vasodilatation of cold limbs. The responses to cooling are indicated both when the nerves to the skin and muscle blood vessels are intact and also when the preparation is acutely denervated.

The purpose of the investigation has been to measure and combine some haemodynamical parameters characterizing arousal from hibernating state. A particular emphasis is laid on the physical factors involved during this impressive natural phenomenon.

Direct techniques for *in vivo* determination of radial distension of aorta and apparent viscosity of blood were not available. Therefore these parameters have been measured by *in vitro* methods in this study.

Material and methods

Hedgehogs, Erinaceus europaeus L., *were kept in single cages with hay in a room kept at* outdoor temperatures. They were fed pellets and water *ad lib.*

Elasticity

The thoracic aorta was excised as quickly as possible, adjacent tissue removed in Ringer solution and the ends of the vessel tied tightly on two cannulas in a temperature bath. One cannula was connected to cylinder with a piston for pressure production and the other cannula to Sanborn strain gauge pressure transducer. As the transmural pressure was increased in small steps to approximately 100 mm Hg/cm² and then reduced in similar steps, the external diameters were determined with an optical micrometer screw. Care was taken that longitudinal strain should not interfere with the radial strain. Measurements were made on 23 vessels short time after sacrificing the hedgehogs. Fh vessels from hibernating animals and five vessels from non hibernating animals were tested at 6, 20 and 34 C. The other vessels were tested at 20 C and at 6 or 34 C according to the actual temperature of the animal.

I ue sity

Blood sampling

Blood was taken ei he by direct punctur of the ascending aorta or when several samples rom the same animal were taken during arousal, by a polyethylene catheter inserted through he carotid artery to the aorta. The catheter glassware and the measuring hamber are siliconized and heparin used as anticoagulant. Viscosity was normally measured short time after sampling or some cases after storage overnight in the old.

Viscosity analysis

A Brookfield micro iscomete (LVT) with a cone plate was employed. The plate could be rotated eight different speeds, representing rates of shear from 230 to 1 15 sec At each speed the rat of shear of the fluid was constant and niform everywhere in the cup.

Joly 1962 has reported possible disturbance from albumin t the bovine serum surface. As described in he esults, serum from hedgehogs showed no increases in iscosity with falling speeds. Because the disturbanc could only be excluded for serum, samples of blood and plasma were usually measured t shear rates from 230 to 5.8 sec and the wo lower speeds ere never used.

All samples were measured at temperatures 6 20 and 34 C. Except hen controlling tests, determinations w (made at the emperature nearest the body temperature. Temperatures in the sampl p ere maintained within ±0 1 C.

Filling of the cup w b 1 ml of solution was performed with are since bubbles ph false alues. Water, water/ethanol nd tandard oil (Brookfield Ltd.) showed no shear dependence and gav readings acc rate) 1 % of the f ll scale. Where shear tress was needed, it was calculated by the form la

Shear stress = % of f ll scale orque 2/3) r r³

Hematocrit w re obtained by rifugation in Wintrobe tubes. *Body temperat* refer to values measured deep in the ck using coppe onstantan element



Fig. 1 Fig. 2

Fig. 1 The relation between external diameter and transmural pressure of the aorta of hedgehog
Δ first series of increasing pressures,
× ○ three repeated series, 20° C

Fig. 2 The relation between external diameter and transmural pressure of the aorta of hibernating hedgehog, t different bath temperatures.

Results

Distensibility of th ort wall

When the transmural pressure was raised for the first time after the extirpation of the vessel, the diameter/pressure diagram obtained bowed a smaller cross-section for the vessel than in successive experiments when the pressure was raised and lowered several times in the same specimen (Fig 1) All these curves almost coincided, ex posing a small hysteresis effect of only 1— mm Hg/cm even for larger steps. Since the aorta was always initially tested for leakage by pressure elevation, the results presented in this report correspond to these later curves. Due to the *i vivo* pressure, however the later curves could as well represent conditions in the living animal

Eff cts of temperatu e

The main effect relating to our problem on elasticity of vessels, was the typical one of temperature on vessel diameter The diagrams of external diameter versus transmural pressure or tension versus radius (Figs 2, 3) showed that the cross-section of the aorta increases with falling temperatures from 34 to 6° C. The increase in diameter corresponds to, and compensates for f ll in pressure of 15—20 mm Hg/cm

It is observed that the elastic properties remain relati ely unchanged. The curves are nearly parallel and do not converge or diverge at lower pressures

Because there is no l near relationsh p between length nd tension, Hook law is not followed (Fig 3) The curves bend towards the axis of tension, mainly at 6° C. Nevertheless for the small ph siological pressure riations t constant temperature the curves re almost linea

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F lling of the cup w h l of solution was performed with care since bubbles gave false values. Wat r, water/ethano l d andard oil (Brookfield Ltd.) showed no shear dependence and ga readings accura th 1 % of the full scale Where shear ress was needed, it was calculated by the formula

Shear stress = % of full sc le que 2/3)π r³

Hematocrits were obtained b rif gation in Wintrobe tubes. *Body temperat* refer to alues measured deep in the k using copper onstantan herm u

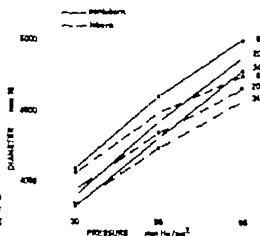


Fig. 5a. The relation between mean values of diameter and pressure of the aorta of hibernating and non-hibernating hedgehogs at three bath temperatures.



Fig. 5b. Young' modulus at P 30—P 90 for different bath temperatures for the same groups as in Fig. 5a.
Fig. 5c. Young modulus t the typical pressures and temperatures found

General viscous propertie of the blood

Unless specified the values given for apparent viscosities were measured at a rate of shear of 115 sec⁻¹ The mean viscosity at 34 C for 59 hedgehogs was 7 4 centipoises (cps) If one wishes to compare this figure with data for other animals, the value corresponds for the same blood to 6 9 cps at 34 C and 5 8 cps at 37 C, at a rate of shear of 230 sec In any case the viscosity of the blood from hedgehogs was high compared to values on most other vertebrates—and to the data obtained by the author concerning blood from other homeotherm animals

Shear stress versus rate of shear reveals curves bending slightly towards the axis of rate of shear (Fig. 6 a) As also shown in Fig 6 b the blood shows pseudoplastic characters by the rising viscosity at falling low rates of shear If the rate of shear was reduced from 115 sec to 23 sec the apparent viscosity rises to approximately double the value In nearly half of the samples tested the plasma showed no shear rate dependence In the other half it showed an increase in viscosity as in blood at low rates of shear

Serum never exhibited non-Newtonian characteristics

Viscosity of blood increased with hematocrits I [illegible] ly at hematocrits higher than ca. 55 [illegible] serum and Ringer solution

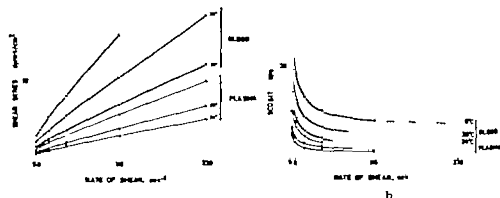


Fig. 6a. Shear stress versus rate of shear for blood and plasma from hedgehog, measured at three bath temperatures 6 20 and 34 C.
Fig. 6b. Relation between the apparent iscosity and rat of shear on the same sample

lower values and showed less shear rate dependence than blood corpuscles suspended in plasma (Fig 8)

Observations in microscope indicated less cell aggregation in blood from cold animals.

Effects of temperatu e

The effects of temperature on apparent viscosity are profound. For all shear rates, the apparent iscosity of a sample increased to about double when temperature dropped from 34 to 6 C (Fig. 6 b) Ho ever the relative viscosity of blood to water was either independent of temperature or there was a ery small increase.

Properties of blood and plasma f om d fferent animal groups

ig. 9 a shows that the mean apparent viscosity at 34 C for blood from non-ating hedgehogs in autumn was somewhat higher than the alues obtained in at the start, during or after arousal from th hibernating state. This tendency as reflected also in the plasma data.

The hematocrites (50 52. 50 50) of the blood tested in the different groups were

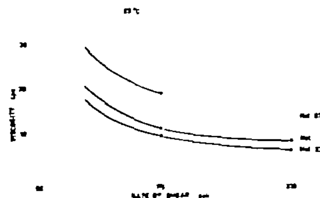


Fig 7 Viscosity ersus rate of shear for blood ith resolved red ells giving different hematocrites. Temperature 20 C

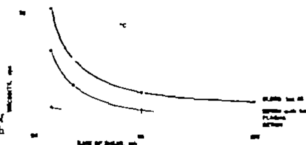


Fig. 8. Viscosity versus rate of shear for plasma and serum with or without resolved red cells.

almost constant—although slightly higher in the hibernating animals—and can hardly explain the difference. (Total protein in plasma measured by the Biuret method was also highest in autumn.)

In relation to our problem, it is more important to compare values measured at the actual temperatures of sampling from the three groups. Start arousal near 6° C, arousal 20° C, and after arousal 34° C. In Fig. 9 b one can observe that the mean viscosity—at the rate of shear 115 sec⁻¹—decreases more during an arousal than would the viscosity of one sample studied at the same temperatures (n = 50). The floating dotted lines in Fig. 9 b represent figures obtained by withdrawal of 1.5 ml blood at all three temperatures from the same arousing animal (n = 7).

Plasma values are given both for shear rates 115 and 230 sec⁻¹.

Discussion

Viscosity

The red cells have great influence on the viscous characteristics of the blood. A large difference was shown between viscosity of serum and plasma—with or without resolved cells. This gives support to the suggestion by Merrill et al. (1963 a) that the fibrinogen molecules may interact in a network involving blood corpuscles. The effects of thrombocytes and coagulation factors are, however, not known. It is

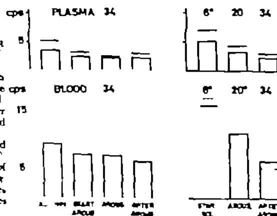


Fig. 9a. Mean apparent viscosity at 34° C for blood and plasma from different groups of hedgehogs (n = 50). Normal non-hibernating animals in autumn. Winter animals short time after the start of arousal, during arousal and after arousal. Data are given for plasma both for rate of shear 230 and 115 sec⁻¹ (floating lines).

Fig. 9b. Viscosity for samples of blood and plasma at 6°, 20° and 34° C measured at the actual temperature of the animal (n = 50) when sampling. The floating dotted lines refer to values valued by taking the three samples from the same animals (n = 7).

interesting that Björck, Johansson and Nilsson (1962) found large variations in fibrinogen content of blood from hedgehogs, particularly in connection with the sudden appearance of shear rate dependence of plasma samples.

Much work has to be performed to explain the functional behaviour of blood in the body but with a shear rate variable viscometer one may have a good starting point. Unfortunately we know little about the shear rates *in vivo* in spite of discoveries such as a plasma zone near the vessel wall and blood cell aggregation at the axis. These effects will be further discussed in a companion paper (Kirkebö, 1968).

Whatever the rate of shear during arousal from hibernation, it must be important that the viscosity is reduced by temperature to values lower than 40 % of the initial.

Hock (1964) found that the relative viscosity of blood of hibernating Arctic ground squirrels was higher than that found in active animals over the same temperature range. The viscosity relative to water increased at low temperatures. These findings are supported only in part by the present results. The highest apparent viscosity were found on active animals in autumn. The relative viscosity of hedgehog blood was almost independent of temperature as also shown by Merrill et al. (1963 b) for human blood. These workers, Merrill et al. (1965) did find experimental pressure flow data in hollow fibers, in agreement with measurements in a rotational viscometer and their predictions based on the assumption that blood flows as a homogenous continuum.

Elastic properties of the vessel wall

As the transmural pressure increases the curves of radius versus tension in the aorta bend towards the axis of tension (Figs. 3, 4). These findings could easily be fitted into the theory of Burton (1954). He has given values of increasing elastic modulus of the four elastic components of the vessel wall: Endothelium, smooth muscle, elastic fiber and collagen fiber working in co-operation. The collagen fibers with a Young modulus of 1 10^9 dynes/cm² comes into action latest. The elastic modulus 1.7—2.9 10^6 dynes/cm² found in the present investigation is close to the values given by Burton for elastic fibers (3 10^6 dynes/cm²). Therefore one might assume that the elastic fibers of aorta in hedgehogs are responsible for most of the volume distensibility in the normal physiological pressure range. Nevertheless Bergel (1961) have suggested that the elastic modulus of vascular smooth muscle (0.1 to 1.5 10^6 dynes/cm²) is much higher than the figure given by Burton (0.06 10^6 dynes/cm²). Bergel's data indicate that Burton's theory may be too simple. Until we know more about the complex properties of smooth muscle conclusions on present results are also uncertain.

Effect of temperature on d t n

The curves at temperatures 34 20 6 C in the elastic diagram are relatively straight and parallel over the whole physiological pressure range.

Want of relevant data makes it difficult to explain the temperature effects on aorta distension as a mere muscular constriction at higher temperatures. Relaxation of smooth muscles in dog arteries at normal temperatures are known to give a lowering muscular elastic modulus (Bergel, 1963). If similar properties are actual in the vessel wall during a temperature distension, a greater change in form of the curves at low pressures might be expected.

From Poiseuille's equation, the hindrance ($H = 8\ L/\pi r^4$) corresponds to the geometrical proportions of flow resistance. The flow is proportional to the fourth power of the radius. If hindrances in the aorta are calculated from the actual mean pressures and diameter found in non-hibernating and hibernating states, the aorta diameter reductions in hibernating animals give an increased hindrance factor of 1.9 times. However if there hypothetically was no distension with falling temperature the increase in hindrance would be 2.5 times. In other words, the temperature effect compensates the rise in hindrance of the aorta by 40 % as compared to the initial value in the non-hibernating state.

The investigation has been supported by grants from L. Meltzers Höyskolefond and from U.S. Navy European Research Contracts Program. The author is indebted to professor dr. Einar Hanssen for the discussions stimulating the author to start this investigation. I wish to thank Anne Kirkebö for her careful work.

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Acta physiol. scand. 1968. 73. 394—406
From the Institute of Physiology University of Bergen, Norway

Cardiovascular Investigations on Hedgehogs during Arousal from the Hibernating State

By

ARNE KIRKEBÖ

Received 17 November 1967

Abstract

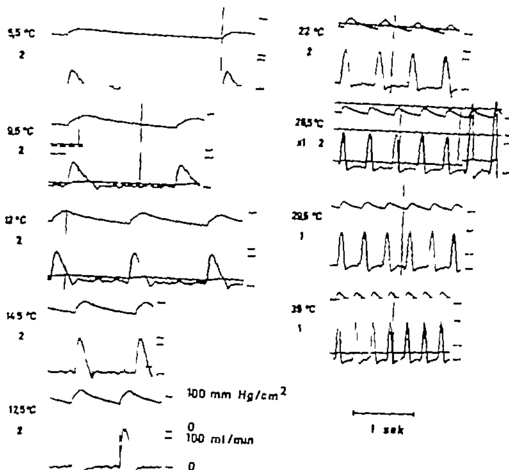
KIRKEBÖ A. *Cardiovascular investigations on hedgehogs during arousal from the hibernating state* Acta physiol. scand. 1968. 73 394—406.

Haemodynamical changes were followed in hedgehogs during arousal from the hibernating state. The temperature in the anterior part of the body increased prior to the temperature in the posterior part. Cardiac output—measured with implanted electromagnetic probes—rose faster than the heart rate in early arousal, but slower in the later phases. Arterial pressure reached maxima at temperat re of 20—30 C i the neck. The decrease in peripheral resistance was greatest in the first part of arousal and was largely due to the fall in blood viscosity with temperature. There is usually no f rther decrease in hindrance abov 15—20 C. The dilation of posterior essels were compensated by constrictions i regions already warmed. Due t the fall in viscosity of blood with temperature, warmer organs are more easily circulated than colder portions. This and other autoregulatory effects of physical nature are discussed.

... ding the lines of Dubois investigations of the previous century Chatfield and ...an (1950) showed that the rise in heart rate of the hibernating hamster followed rise in body temperature during arousal, and that the anterior portions of the body warmed faster than the posterior portions. (Lyman and Chatfield 1950)

Their finding that the increasing blood circulation was confined to the fore part during most of the arousal periods was confirmed by Bullard (1964) and Johansen (1961) by the fra tional distribution of radioactive indicators. Bullard found no evidence for a complete cessation of flow in the hind part from a ma vasoconstric tion of the posterior vasculature No shifts of blood vol ıme of haemodynamic significance could be d mon trated in the ground squirrel.

Blood pressure has been measured during arousal in hamsters (*Mesocricetus auratus*) (Chatfield and L man 1950) in ground squ irrels (*C itellus tridecemlineatus*) (Lyman and O Brien 1960) and in hedgehogs (*Erinaceus europaeus*) (Eliassen 1960) In the begi ning of rousal all the species showed a rapid pressure increase which reached a maxim m alue long before the heart was beating at its fastest rate Cardiac output wa never det rmined, but the peripheral resistance wa



1 Instantaneous flow and pressure ecords measured on the aorta during arousal from hibernating stat 5 C to an active state at 35 C. From 26.5 C the gnal amplification ar educed f om 2 to 1 Calibration marks on the right side of each r

uon is reduced from 2 to 1 One can also observe a rise of blood pressures and a more rapid fall d ring diastole w th increasi g temperatures which were most pronounced in th arlier part of arousal.

Fig 2a show the parameters measured as a function of time in one typical hedgehog during rousal f om the hibernat ng stat

T mperatur

The temperature in th thor egi n ncreases before the temperat re n the rectum. The fastest rise in temper t w found during the middle part of arousal. When the environmental temperat w e low as here th two temperatures diverged until the thorax region nearly hed it normal temperat re of 31—35 C in the non-hibernating state The abd inal temperat re then ncreased r pidly in the final phase.

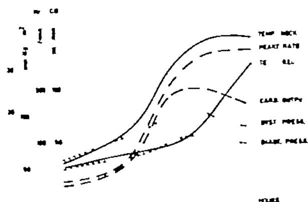


Fig. 2 a. Relation between time and temperatures, arterial pressures, cardiac output, heart rate measured on one hedgehog during arousal from hibernation.

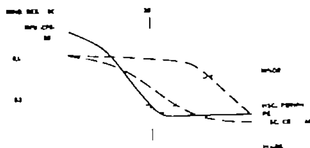


Fig. 2 b. The corresponding total peripheral resistance, hindrance and blood viscosity determined on the same hedgehog. The viscosities were found by blood sampling at 7 C, 20 C, 35 C from the same animal and refer to the temperatures in the neck and rectum.

P ressure

Systolic and diastolic blood pressures were relatively high at hibernating temperatures. 24 over 14 mm Hg/cm² were the lowest pressures measured even on quiet hibernating animals with heart rates lower than 20 beats/min (to 7). The mean values at 6° C after start of arousal were 40 over 27 mm Hg/cm² as shown in Fig 3 a.

Fig. 3 a gives the mean values on 23 animals followed through the whole temperature scale from 6° to 35 C as a function of the temperature in the neck. The systolic, the diastolic and pulse pressures all grow fast and reach a maximum at 20—30 C in the thorax region. Thereafter a small decline was usual. (Fig 2 a, 3 a)

H art te

The heart rate rose evenly from few to 250 be ts per min te and seems for undisturbed steady arousals to be the parameter most dependent on the temperature of the thorax. The most rapid increase in heart rat was observed during the middle phase of arousal.

Cardiac output

followed heart rate in the first part of arousal increased faster than the heart rate due to a slowly increasing strok d me F g 2 b. However fo

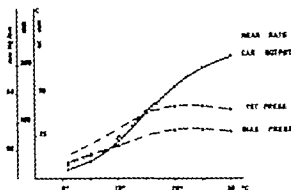


Fig. 3 a. Relation between anterior temperature (neck) during arousal and mean values (n = 23) of heart rate, cardiac output and pressures in the aorta of the hedgehogs.

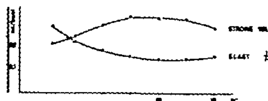


Fig. 3 b. Changes in the corresponding mean stroke volume and the relative hindrance calculated from the mean diameter of the aorta at the actual pressures and temperatures.

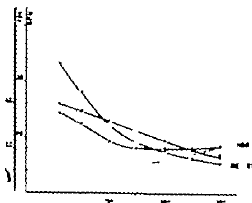


Fig. 3 c. Anterior temperature versus the corresponding mean values of total peripheral resistance, and of hindrance calculated from cardiac outputs, pressures and viscosities at shear rate 113 sec⁻¹ given for the actual central temperatures. The stippled lines refer to the viscosity (upper line) and the hindrance for rates of shear rising from 23 sec⁻¹ to 113 sec⁻¹ during arousal.

rates higher than 200 beats/min the stroke volume decreased. In some animals, as in Fig. 2 a, the cardiac output fell in spite of a further rise of heart rate. Flow rates less than 1 ml/min were observed during the hibernating state and even at the start of the arousal.

Peripheral resistance

Variations with time of total peripheral resistance (R = driving pressure/flow) calculated on a single hedgehog are given in Fig. 2 b. The curve accentuates the changes also evident in Fig. 3 c by the mean values versus temperature. The peripheral resistance fell sharply during the first phase of arousal. Above a thoracic temperature of 20° C, the total resistance was relatively constant or did not fall any more than the viscosity of the blood.

Though we may find similar values at 20° C and at 34° C, the situation during the middle phase of arousal is probably characterized by a sharper distinction between a fast circulating and a slowly circulating blood volume.

Because pressure, heart rate, stroke volume and flow velocity all increase, the work per unit time of the heart must increase faster than any of the single parameters, though we find a decreasing resistance.

Viscosity

The apparent viscosity of the blood, determined for the actual temperatures in thorax during arousal (Kirkebö 1968) is plotted in Figs. 2 b, 3 c. It is observed that the fall in viscosity explains the greatest part of the fall in total peripheral resistance. Bullard (1955) presents a similar conclusion on the importance of viscosity in the hypothermic rat. Fig. 2 b also gives the viscosity representing peripheral vascular regions with the same temperatures as in the rectum. If the *in vitro* data (rate of shear 115 sec⁻¹) are also valid for the *in vivo* condition, the viscosity values of blood must be found between the two curves. The lower curve should be the important one, because the blood circulated through the heart and the vital central organs are believed *to have temperatures near to this. A large difference in blood viscosity must* exist between the peripheral and the central regions during the middle active period of arousal.

Viscosity dependence on rate of shear

Supposing Newtonian flow (F) which is probably only valid for high flow velocities (v) the rate of shear near to the vessel wall (radius R) can be calculated from the formula.

$$(dv/dr)_R = 4v/R = 4F/\pi R$$

Using the mean flow and mean internal radius determined in vitro, at the pressures and temperatures of start and end of arousal one can estimate the rate of shear after arousal to be 200 sec [illegible] t the wall of the aorta. Due to the long diastoles in the hibernating state, the flow in the aorta for single heart strokes is assumed to finish within one second (Fig. 1). This will give rate of shear of 55 sec during systole. For the moment of maximal velocity during the systole this figure is too low. On the other hand, the rate of shear falls towards zero in the central part of the artery giving a *reduction of 2/3 in* the mean rate of shear across the vessel

(From integration of 1 R R∫O d /dr [illegible] dr)

For lack of reliable data on circulation in the more important resistance vessels, estimates of rate of shear [illegible] th arterioles in literature are very dissimilar (Haynes and Burton 1959, Wells and Merrill 196[illegible]). In order to get an idea of the effect of shear rate dependent [illegible] during arousal, the fall in mean blood viscosity (n = [illegible]0+7) with temperature and rise in rate of shear from 23 sec to 115 sec are shown in Fig. 4. *It is seen that the decrease in viscosity is much greater than* [illegible] ed by the curv[illegible] rate of shear (Kirkebö 1968)

While bearing in mind the possible limitations of *in vitro* data the resulting viscosity curve is transferred to Fig. 3 c. (Upper stippled line) The true viscosity value of blood in the aorta is found probably somewhere between the two viscosity curves. It is demonstrated, nevertheless, that in cold, slow acting animals, flow velocities lower than certain minima may give circulatory problems from high viscosity. The relatively high pressures observed may reflect the same conditions.

Hindrance

Resistance to flow in Poiseuille's law is made up of two distinct factors, viscosity and hindrance. The hindrance ($H = 8L/\pi R$) refers to the resistance from the geometry of the tubings. It is calculated for the animals in question (Figs. 2, 3 c, 6 b) by dividing total peripheral resistance by the actual viscosity. The mean total hindrance falls while the arousal speeds up. During the first 15 C of arousal the changes in hindrance and viscosity usually contribute the same amount to the reduction in peripheral resistance. For the rest of the arousal, the viscosity of the blood explains the whole fall in resistance. The hindrance is usually relatively constant, but may show large variations.

During the first period of arousal there must be an extending dilatation in the thorax region. The dilatation of the abdominal regions above 20 C must usually be compensated by an corresponding constriction probably in the central body.

Fig. 2 b shows a rise in hindrance at later arousal as a consequence of constriction. In this special animal there was no net reduction in hindrance. The fall in net resistance was only due to the change in viscosity.

A hindrance curve corresponding to the viscosity going through rates of shear from 23 to 115 sec is also given in Fig. 3 c (Lower stippled line). If the viscosity measured at 115 sec is lower than the actual values at low temperatures, due to smaller flow velocities, the hindrance may show nearly constant values also in the earliest period of arousal.

Elasticity

As pointed out in a companion paper (Kirkebo 1968) we know little about the passive elastic effects from the transmural pressure on the arterioles.

The importance of the elasticity of vessel wall on hindrance can, unfortunately only be given for the aorta after *in vivo* measurements of radius.

The values of relative hindrance (k/r) are calculated for the mean pressures at each temperature found during arousal. The curve shown (Fig. 3 b is remarkably like the curve of hindrance calculated from the flow, pressure and viscosity data, but no conclusions should be drawn.

Variability and regulations of the circulation

Cardiac output is the parameter measured that makes the most profound and sudden variations. The deep respiratory inspirations, for instance have a highly stimulating action on flow particularly observed here in the carotid artery of 1 mm diameter

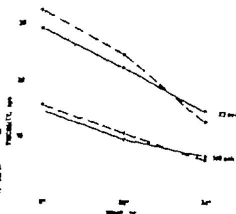


Fig. 4 Relation between temperature and mean viscosity of blood at rates of shear 23 sec and 115 sec The long stippled lines give viscosities gained by taking three samples from the same animals during arousal. The dotted line refers to the viscosities estimated for rate of shear rising with temperature from 23 sec to 115 sec

(Fig. 5) But sudden changes can appear without any other influences. In Fig 6 a, b, cardiac output shows an intricate pattern in spite of relatively steady rates of pressure and heart rate. It is difficult to know whether the cause lay in the heart efficiency peripheral regulations or venous capacity The curves of peripheral resistance and hindrance must also show corresponding variations. They also show an example of a fall in hindrance during the late period of arousal which may sometimes occur

It is clear from the observations on a series of arousals (n = 50) that there are many routes going from the hibernating state to an active homeotherm life at 34 C. In two hedgehogs woken from hibernation on two successive days, the second arousal in a warm room gave much smaller cardiac output in spite of an absence of change in the mean blood pressure heart rate and viscosity with temperature

Fig. 6 a further show that sometimes the rise in rectal temperature may show a sudden increase in rate. From the actual recordings this is seen to occur at a temperature difference of 25 C a short time after an increase in flow and decrease of arterial blood pressure had ceased Venous pulse pressures were larger during the change.

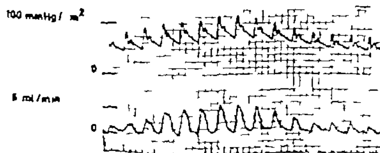


Fig. 5. The influence of respiratory rhythm demonstrated on aorta pressure and aortic during one expiratory cycle Stroke volumes from to 13 al

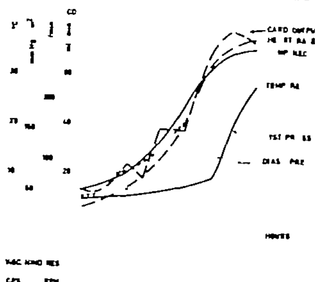


Fig. 6 a. Heart rate arterial pressures and temperatures in neck and rectum eruus time during arousal of one hedgehog. Sudden hanges are show for cardiac output, and for rectal temperatures t 9 C.



Fig. 6 b. The corresponding total peripheral resistance hindrance and blood iscouty at central anterior regions.

This rapid opening of the abdominal essels cannot be due to a transmural stretching, since the pressure was already high more than half an hour before. The fall in viscosity with temperature can explain changes in blood flow and temperature in a slowly moving front This may gradually extend the warmer region by heat transport through the tissue and by residual flow in colder organs But in this particular arousal, the explanation most reasonable is a dilatation of the sphincters and resistance essels in the abdominal body by nervous regulations.

A nervous asomotor control is in agreement with the conclusion of Lyman and O'Brien (1960) based on their excellent investigations using pharmacological agents. They suppose the arousal to be maximal, since injection of norepinephrine will not further accelerate the rapidly beating heart. (Lyman and O Brien 1963)

For very small dist rbances, the hedgehog nevertheless, seems to be able to further stimulate its heart for a short time Larger disturbances, even touching the animal can have a profound and retarding effect on the regulatory balance of the arousal. If the hedgehog is stretched on the animal board after the temperature difference between neck and rect m is established (Fig. 8) the temperature of the thorax suddenly declines while the bdominal temperature begins to rise rapidly.

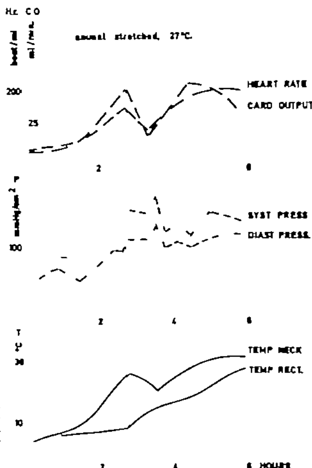


Fig. 7 Cardiac output, heart rate, arterial pressures and temperatures in anterior and posterior regions—prior to and after hedgehog had been suddenly stretched on the animal board.

The blood pressures may remain constant if the normal alue is reached, but heart rate and most important, the cardiac output, are drastically reduced for a long time. The fluctuating cardiac outputs shown thereafter are often observed when th- temperature differences are small.

Experimental handling of the animal and injectio of asomotor drug affecting the heart, may give temperatur changes due to lower cardiac output and heat production—besides the peripheral asoregulating effects examined.

With the intention of testing wh ther t was possible to produce a udden asodilatation of the hindpart papa erine was injected through the pressure tubing. The results were, however masked by a h a y red ction of cardiac output. Both temperatures ceased to rise. On the ther hand when isoprenaline was used as a heart stimulant simultaneously w th the papa erine cardiac output continued to rise, while heart rat and pressure were distinctl educed (Fig 9) Here a sudd rise in rectal temperature was evident



Fig. 8. Cardiac output, heart rate, arterial pressures and temperature in anterior and posterior regions prior to and after vasodilatation produced by papaverine. Isoprenaline was given in order to stimulate the heart activity.

In spite of a continued vasodilatation, seen from the low diastolic pressures and the short lasting systolic peaks after a time the decrease in difference between the two temperatures ceased at a difference of 12—15 C. The conclusion again seems to be that a vasomotor dilatation in the abdominal region may be effected by humeral (and neural) influences but that an autoregulatory effect of the low temperature itself promotes a full blood flow in the colder organs.

It is known from other mammals, that the sensitivity of adrenergic transmitter on vascular smooth muscles are reduced by cooling. (Folkow *et al.* 1963)

Discussion

Temperature, heart rate, blood pressure

The temperature in the anterior part of the body increased prior to the temperature in the posterior part, as shown also in the arousing hamster by Lyman and Chatfield (1950). The heart rate rose most rapidly during the middle period of arousal—as did the thorax temperature (Fig. 3 a). This is in accordance with the findings of Chatfield and Lyman (1950) and Eliassen (1960). The present work confirms also the observation of these authors that maximum blood pressure was reached long

before the heart was beating at its highest rate. Eliassen relates his data to the rectal temperatures of hedgehogs arousing at room temperature, while the present res lts refer to deep neck temperatures of animals arousing in a cold room.

Cardiac output

The difference in cardiac output (measured by the Fick principle) of euthermic and hibernating ground squirrels found by Popovic (1964) was of the same order as in the present investigation. But in the hedgehogs, no important change in hematocrit was found that could seriously affect the peripheral resistance.

Bullard (1964) found that the stroke volume in thirteen-lined ground squirrels showed no consistent change with a change in thoracic temperature or heart rate. In the present work on hedgehogs it was shown that cardiac output increases a little faster than the heart rate in early arousal. A small decline in stroke volume is found in the latest phase of arousal (Fig. 3 b)

Peripheral resistan e viscosity and hind ance

The measurements of cardiac output and viscosity have given a better basis for estimating the peripheral resistance and hindrance.

The total peripheral resistance is falling strongly especially during the first half of arousal (Fig. 3 c) The fall is largely due to the decrease in blood viscosity with temperature. The apparent viscosity of the blood in thorax decreases nearly propor tionally to the increase in temperature The hindrance usually shows a fall only during the early phases of arousal. Possibly t is partly due to a passive distension of vessels with rising pressure.

The metabolic heat production and the temperature are higher in the central part of the body The blood will more easily circulate the warmer portions due to the difference in blood viscosity between the peripheral regions and the central regions. There must be an important autoregulatory effect giving 2—3 times higher local resistance in the cold and slowly circulated peripheral organs (Fig 2 b, 3 c) Specially during the middle acti e period of arousal, this effect will facilitate the blood flow to the central organs, already warmer and more richly supplied by blood.

The decrease in viscosity with increasing rate of shear during arousal will contribute with a similar facilitation. Flow velocities lower than certain minima in the cold tissues may giv circulatory problems from high iscosity

Since the curves show no discrepancy in iscosity for a temperature effect at the start (as they do at the end of arousal) this autoregulatory mechanism is hardly the usual cause of division of the circulation of the body i to two compartments. Likely primary reasons may be higher pressure and nervous or metabolic regulations. Organs with higher metabolic prod ction of heat and vasometabolites will immediately take advantage of a falling iscosity Bullard (1964) found a quit similar distribution of flow in control animals and in those deeply hibernating or in early arousal.

Lyman and Chatfield (1950) Johansen (1961) and Bullard (1964) f

progressive vasodilatation during arousal in the order of thorax, anterior portions and lastly posterior portions of the body.

In the present investigations the hindrance was shown usually to decrease only during the first 15° C of arousal. For the rest of arousal, the hindrance was normally constant, although large variations occurred in different animals. The meaning of these results must be that during the first period of arousal there must be an extending dilatation in the thorax region. But above 20° C the sudden, or evenly developing dilatation of the abdominal and other peripheral warming regions, must usually be compensated by a corresponding constriction probably in the central body. This part is already warm and may reduce its metabolic rise. Such a reduction in central organ flow is not supported by Bullard (1964) unless one assumes a falling stroke volume at the end of arousal, as in the present finding on hedgehogs. His control group of awake thirteen-lined ground squirrels may also reflect another physiological condition than do the hedgehogs just aroused.

An opening of peripheral vessels can occur suddenly and must most likely also be under nervous control.

The author is indebted to the U.S. N y European Research Contracts Program and to Nasjonalforeningen Det Norske Råd for Hjerte og Karsykdommer supporting the present investigation.

Gratitude is due to professor dr Elna Eliasson for his stimulating interests in my ork and for ha ing placed the necessary facilities at my disposal.

I am also indebted to Aagot Kirkebö for her technical skill.

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Metabolic Effects of Glucose Infusion in Man

By

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Abstract

Svedmyr, N *Metabolic eff cts of glucose infusion in man.* Acta physiol. scand. 1968 73 407—410.

In experiments on man the potential importance of the hyperglycemic effect of adrenaline for its calorigenic action was studied. Glucose was infused in dose of 0.005 g/kg/min for 30 min total dose 22 g) The concentration of glucose in blood increased little more 78±4.2 mg/100 ml) than after an infusion of adrenaline in dose of 0.10 μg/kg/min for 30 min 154±4.3 mg/100 ml) The oxygen consumption was not stimulated during or after the glucose infusion. The CO_2-elimination and the respiratory quotient (RQ) increased, however The free fatty acid content in plasma decreased but the lactate concentration in the blood was not altered. It is probable that the hyperglycemic effect of adrenaline is of no importance for its calorigenic action.

In previous experiments in man (Svedmyr 1966 a, b. Svedmyr and Lundholm 1967) the importance of the mobilizing action of adrenaline on free fatty acids (FFA) and lactate for its calorigenic effect was studied. When the FFA mobilizing effect of adrenaline was selectively blocked w th nicotinic cid, the calorigenic effect was reduced by about 30 %. From the relationship between the lactate increase in the blood and the increase in oxygen consumption after infusion of lactate in a dose hich ga approximately the same lactate increase as after adrenaline infusion, it was estimated that about 35 % of the calorigenic effect of the infused adrenaline could be assumed to be due to its lactate-increasing effect. These calorigenic effects were believed to be caused chiefly by different energy-consuming resynthesizing proc esses which follow after an increase in the concentrations of lactate and FFA in the blood. It is considered that the liver plays a major role in these processes.

The remaining part of the calorigenic effect of infused adrenaline, i.e. about 35 % assumed to be mainly due to the cardioaccelerator effect of the adrenaline, to direct stimulation of the oxygen consumption of skeletal muscle and adi and possibly to the hyperglycemic effect of the adrenalin (S r 1 I the present investigation the importance of the hyperglyc t w

Methods

The experiments wer performed on 5 healthy male subjects of ages 25—31 years, 4 of whom had also taken part in the adrenaline experiments (Svedmyr 1966 a). As in this latter study the experiments were carried out in the morning, the subject having fasted since the evening before. A room thermostatically controlled at 4.0 ± 0.5 C was used for the experiments. A plastic catheter was inserted int the cubital ein of each arm, one for taking blood samples and the other for infusion of glucose when the catheters were not being used for these purposes, 0.9 % NaCl solution without addition of heparin was infused through them. After the preliminary preparations, w th commencement of the NaCl infusions, the subject rested for 60 min, after which the basal metabolism was determined during two 10 min periods. During each of these periods a basal blood sample was taken, the mean alues obtained from these samples being taken as basal alues. The infusion sol tion in one arm was then changed to glucose, which was infused in total dose of 22 g over a period of 30 min (0.003 g/kg/min). The oxygen consumption, CO_2 elimination and entilation were measured w th Hartmann and Braun metabolic recorder during the periods 0—10 25—35 45—48, 55—65 85—95 and 115—125 min after the start of the glucose infusion. During each of these periods blood samples (7 ml) were taken for determination of lactate, glucose and FFA. The blood pressure as measured by auscultation and the pulse counted, at 5—10 min intervals. The blood lactate concentration was determined according to Lundholm, Mohme-Lundholm and Vamos (1963) blood glucose according to Bergmeyer and Bernt (1962) and plasma FFA according to Trout, Estes and Friedberg (1960).

Results

During the glucose infusion the blood glucose increased, reaching a maximum (+78 ± 4.2 mg/100 ml, P < 0.001) at the end of the infusion and then falling again to the initial level, which was regained after approximately 90 min (Fig 1). The corresponding value after infusion of adrenaline (0.1 μg/kg min for 30 min) was 54 ± 4.3 mg 100 ml (Svedmyr 1966 a). The oxygen consumption showed a slight tendency to decrease (−0.050 ± 0.045) but this effect was not significant. The CO_2

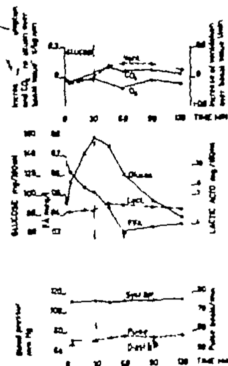


Fig 1. Effects of glucose infused i. in total dose of 22 g for 30 min.

TABLE 1 The influence of glucose infusion on the glucose concentration in the blood and on oxygen consumption. Mean of 5 tests ± SEM Glucose was infused i.v. for 30 min in total dose of 22 g P = probability that the effect was due to chance.

	Glucose mg/100 ml		O_2 consumption ml/kg/min
Basal values	80±7 7		3.46±0.161
Mean change from basal values after			
5 min	34±3.7 P<0.001	0—10 min	−0.036±0.0490
20	63±6.3 P<0.001		
30	78±4.2 P<0.001	25—35	−0.018±0.0370
45	69±5.6 P<0.001	43—48	−0.062±0.066
60	41±4.3 P<0.001	55—65	−0.112±0.032
90	14±3.4	85—95	−0.030±0.043
120	−3±3.5	115—125	−0.084±0.058
		0—90	−0.950±0.045

elimination increased slightly so that the respiratory quotient rose. The ventilation increased parallel with the CO_2 elimination (Fig 1)

The plasma FFA concentration diminished by a maximum of 354±97 meq l, this decrease being significant (P < 0.002 (Fig. 1) The blood lactate concentration rose negligibly The pulse rate and blood pressure remained unchanged, on the whole, throughout the experiment (Fig. 1)

Discussion

In these experiments no significant change in the oxygen consumption was observed during infusion of glucose in a dose which gave a somewhat larger increase of the blood glucose than that observed previously with infusion of adrenaline in a dose of (0.1 μg kg min during 30 min) (Svedmyr 1966 a) (78±4 2 resp. 54±4.3 mg/100 ml) Boothby and Sandiford (1923) reported an increase of approximately 5 % in the oxygen consumption after a considerably larger increase of the blood glucose had been induced by i.v glucose infusion

The increase in the respiratory quotient indicates that the infused glucose was metabolized in the place of other substrates, but without any change in the total oxygen consumption. It is well known that there is an inverse relationship between, for example, the utilization of FFA and of glucose in cardiac muscle (Opie Evans and Shipp 1961 Randle 1963) Nicotinic acid greatly reduces the plasma FFA concentration without influencing the basal oxygen consumption instead the respiratory quotient increase as a sign of increased oxidation of carbohydrates and or proteins (review Carlson 1966, Svedmyr 1966 It seems thus very probable that the hyperglycemic effect of adrenaline is of no or very little importance for its calorigenic action.

Simultaneous with the increase in blood glucose, the plasma FFA diminished. This may possibly have been due to a decrease in the liberation of FFA from adipose tissues and/or to increased elimination from the circulatory system, among other things as a result of reesterification of FFA. A diminution of the plasma FFA concentration during glucose infusion was demonstrated by Dole as early as in 1956. Kjellberg and Östman (1967) have recently shown in man that glucose infusion reduces the liberation of both FFA and glycerol, which may indicate that it is the liberation and not the reesterification of FFA which is affected first and foremost. This may be due conceivably to the increased liberation of endogenous insulin which occurs on infusion of glucose and which results in diminution of lipolysis of the triglycerides of adipose tissue. Butcher *et al.* (1966) have shown that insulin inhibits the stimulatory effect of the catecholamines on the formation of cyclic 3′—5′ AMP in adipose tissue. It is possible, therefore, that the hyperglycemia may increase the liberation of insulin and thereby reduce the concentration of cyclic 3′—5′ AMP in adipose tissue, thus influencing the liberation of FFA. The reesterification of FFA is further an energy-consuming process (Ball and Jungas 1961) and if the glucose infusion had increased the reesterification of FFA, the basal oxygen consumption ought to have increased, which it did not.

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Metabolism of Dopamine and Noradrenaline in Rabbit Caudate Nucleus *in vitro*

By

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Abstract

JONASON, J and C. O RUTLEDGE. Metabolism of dopamine and noradrenaline in rabbit caudate nucleus *in vitro* Acta physiol. scand. 1968. 73 411—417

Slices of rabbit caudate nuclei were incubated with labeled dopamine or noradrenaline. It was found that the catabolism of dopamine proceeded at rate higher than that of noradrenaline. Deamination of both amines was the major pathway of catabolism with O-methylation playing relatively minor role. This was detected by isolating and quantitating each of the know deaminated and O-methylated products of each amine. Pretreatment of rabbits with protriptyline did not inhibit the amount of catabolites formed or the amount of amines retained by the tissue. Synthesis of noradrenaline from dopamine could not be detected in this preparation. The results of this study indicate that the dopamine containing neurons of the caudate nucleus differ in some of their properties from the noradrenaline containing neurons in the cerebral cortex.

It has recently been demonstrated that the relati ely high concentration of dopamine (DA) which occurs in the caudate nucleus (Bertler and Rosengren 1959 Bertler 1961) is localized in a fine system of nerv terminals (Dahlström and Fuxe 1964 Fuxe, Hökfelt and Nilsson 1964) It has also been shown that the cell bodies of these terminals are located in the substantia nigra region. This system of neurons has been termed the nigro-striatal dopamine neuron system (Andén *et al.* 1964 Andén *et al.* 1965) Hamberger and co-workers ha demonstrated that the neurons of the DA ystem ha e uptake properties which are quite different from those of the noradrenaline (NA) containing neurons which occur other regions of the brain (Carlsson *et al.* 1966, Hamberger 1967) When slices of rious parts of the brain are incubated with various catecholam nes these in est gators found that antidepressive drugs such as desmethylimipramine and protriptyline (PTP) were able to inhibit the uptake of amines at the cell membrane in parts of the brain which contain predominantly noradrenaline neurons but had no effect on the neurons in the caudate nucleus containing predc nantl dopamine neurons

In recent studies from this laborat ry th cat bol sm of techolamines has been

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studied by incubating cortex slices (containing predominantly NA neurons) with radioactive DA and NA and the metabolic products were isolated and quantitated (Rutledge and Jonason 1967 Jonason and Rutledge 1968) This present study was conducted to determine whether the DA neurons in the caudate nucleus differ not only in their uptake properties from the cortical NA neurons but also in their ability to synthesize NA and to metabolize both NA and DA.

Methods

Procedures in this study have been described in detail in a previous publication (Rutledge and Jonason 1967) and thus will be presented here only in general terms. Slices of both caudate nuclei from a single rabbit were made and inserted into a single incubation flask containing 5 ml of Krebs-Henseleit solution. Some of the rabbits were pretreated with either 1 or 10 mg/kg PTP 45 min prior to death. Each flask was preincubated for 10 min in a 95 % O_2—5 % CO_2 atmosphere Then, 5.760×10^{-1}[illegible] moles of either C^{14}-DA (27.5 × 10[illegible] curies) or H^3-NA (5.27×10^{-7} curies) were added and the flasks were incubated an additional 30 min. The incubation was terminated by the addition of 2 ml of 2 N HCl. When the retention of the amines was measured the slices were blotted and dried upon filter paper after the 30 min incubation and incubated an additional 10 min in substrate free media. The slices were blotted and dried again and the total radioactivity retained by the slices was determined.

NA and DA were isolated by adsorption onto Dowex-50 column. Normetanephrine (NM) and methoxytyramine (MTA) were first passed over alumina where the two catecholamines, DA and NA, were adsorbed. The alumina effluent containing NM and MTA was then passed over a Dowex-50 column and the two amines were eluted in separate fractions. When the total of the deaminated catabolites was determined, the extract was passed over Dowex and the catabolites in the effluent were extracted with ether The ether volume was reduced to dryness and the radioactivity determined by liquid scintillation counting techniques. When each of the deaminated catabolites was determined separately the Dowex effluent was passed over alumina. The O-methylated compounds namely vanilmandelic acid (VMA) homovanillic acid (HVA) 3-methoxy-4-hydroxyphenylglycol (MOPEG) and 3-methoxy-4-hydroxyphenylethanol (MOPET) passed through in the effluent The catechol compounds namely 3,4-dihydroxyphenylacetic acid (DOPAC) 3,4-dihydroxyphenylethanol (DOPET) and 3,4-di[illegible]phenylglycol (DOPEG) were eluted with 1.0 N acetic acid and 3,4-dihydroxymandelic (DOMA) was eluted with 0.2 N HCl. The catabolites were extracted into ethyl acetate the volumes were reduced for either paper or thin layer chromatography The compounds eluted from the chromatograms with 0.2 N acetic acid Recoveries were determined in each sample by adding carrier substances immediately upon termination of the incubation and quantitating the amount remaining in the eluate after the separation procedures The amount of amines recovered was quantitated by the fluorescence emitted at 335 mμ upon activation at 280 mμ. The amount of deaminated catabolites was quantitated by the phenol reaction of Barness *et al* (1963) The radioactivity of each of the amines and catabolites was determined by liquid scintillation counting techniques in a Packard Tricarb scintillation apparatus.

The values of DA, NA and the individual catabolites from the tissue plus the media were calculated in terms of moles × 10[illegible] per flask obtained after a 30 min incubation period. These values were calculated by correcting for efficiency recovery aliquot factors, and the specific activity of the precursor The values of the combined acid and neutral catabolites as well as the values for the retention of the amines were calculated in the same manner with the exception that no recovery values could be assessed thus no correction for this variable was made. It was assumed that the recoveries of these two groups of compounds was relatively constant. Results are generally expressed as the mean ± standard error of the mean (S.E.M.). Statistical calculations were performed according to Snedecor (1956)

Results

Metabolism of DA and NA normally and after PTP pretreatment

Incubation of slices from rabbit caudate nuclei with DA resulted in the formation of acid and neutral catabolites in amounts which totaled about 30 per cent of the DA added (See Table I) When the slices were incubated with the same conc[illegible]

TABLE I. Retention and catabolism of C DA in caudate nucleus slices from normal and protriptyline (PTP) pretreated rabbits.

Slices of caudate nuclei from normal and PTP pretreated rabbits were incubated for 30 min. with 5,760 × 10^{-10} moles of C^{14}-DA (27.5 × 10^{-4} curies) PTP was injected 1 45 min prior to killing the animal. Control corresponds to samples in which 2 ml of 2 N HCl were added to the incubation fluid before the incubation. Recovery of noradrenaline (NA) equals 68.4 % ± 3.7 and that of dopamine (DA) equals 67 4 % ± 4 0. The NA and DA values but not the values of acid and neutral catabolites were corrected for recovery n represents the number of experiments. S.E.M. represents the standard error of the mean. The values are presented as moles × 10^{-12} The values of NA, DA and acid and neutral catabolites represent amine or catabolite in the media plus that in the tissue. The retention values represent the amounts of amines (or catabolites) retained by the slices after 10 min postincubation in substrate-free-medium. 1 × 10^{-10} moles are approximately equivalent to 5 counts per minutes above background in the original sample assuming 100 % recovery

Treatment		C NA	C[1] DA	Acid and Neutral Catabolites	Retention of C^{14} DA
Normal	Mean	18.7	3,220	1,060	279
	S.E.M.	2.2	394	59	74
		4	4	4	2
PTP 1 mg/kg	Mean	13.3	3,200	1 750	482
	S.E.M.	2.6	240	287	44
	n	4	4	4	3
PTP 10 mg/kg	Mean	13.8	2,640	1 710	595
	S.E.M.	3.9	142	145	96
		4	6	4	3
Control	Mean	18.5	4,610	5	21
	S.E.M.	1 7	896	2	2
	n	2	3	3	2

tration of NA much smaller amounts of acid and neutral catabolites could be detected (See Table II) This is partly d to th fact that NA catabolites are extracted into ether to a much smaller extent than the DA catabolites. However it is evident that DA is metabolized to degree of t least two times that of NA since the difference between the co trol alues and the experimental values of unmetabolized amine are more th n two times higher for DA than for NA (See Table I and II) Furthermore if the ndi idual metabolites in Tables III and IV are summed it is evident that th catabol tes of DA re more than two times that of NA.

From Table I and II t can also be seen that pretreatment of the animals w th PTP 1 or 10 mg/kg did not alter the t tal amo t of catabolic products of either DA or NA. The amount of unmetabolized DA or NA was also not affected by PTP pretreatment.

TABLE II. Retention and catabolism of H NA in caudate nucleus slices from normal and protriptyline (PTP) pretreated rabbits.

Slices of caudate nuclei from normal and PTP pretreated-rabbits were incubated th 5.760 × 10⁻ moles of H NA (5.27 or 7.12 × 10⁻ curies). PTP was injected 1 43 min prior to killing the animals. Recovery of noradrenaline (NA) was 65.4 ±2.1. The NA values but not the values of acid and neutral catabolites and retention were corrected for recovery. 1 × 10⁻¹⁴ moles ar pproximately equivalent to 12 counts per min above background in the original sample assuming 100 % recovery. For further explanation see Table I

Treatment		H NA	Acid and Neutral Catabolites	Retention of H NA
	Mean	3,940	153	457
Normal	S.E.M.	162	51	34
	n	3	3	2
	Mean	3,550	129	518
PTP	S.E.M.	159	44	46
10 mg/kg	n	4	4	3
	Mean	4,380	10.0	50
Control	S.E.M.	158	1.4	23
	n	4	2	3

From Table I it can be seen that very little if any NA was synthesized from DA in rabbit caudate nuclei preparations. When the NA fraction from the Dowex column was collected in 2 ml fractions no definite radioactive peak could be correlated with the fluorescence peak of NA added as a carrier substance. If one sums individual catabolites of newly formed NA as found in Table III it can be seen this sum is quite low especially if one subtracts the control values. This indicates NA was not formed and catabolized in large quantities.

TABLE III. Detailed metabolism of C¹⁴ DA in slices of rabbit caudate nuclei.

Slices of caudate nuclei were incubated with 5,760 × 10 moles of C¹⁴-DA (27.3 10 curies). Mean recoveries of the metabolites were: dopamine (DA) 67.4 ±4.0, 3,4-dihydroxyphenylacetic acid (DOPAC) 22.9 ±4.7 homovanillic acid (HVA) 36.3 ±6.9 methoxytyramine (MTA) 94.2 ±2.1 3,4-dihydroxyphenylethanol

Treatment		DA	DOPAC	HVA	MTA	DOPET
Normal	Mean	3,220	1,500	110	31.3	122
	S.E.M.	394	394	21	1.3	40
	n	4	3	3	3	2
Control	Mean	4,610	7.9	5.2	24.6	5.6
	S.E.M.	896	1.8	2.0	7.6	3.1
	n	3	3	3	2	2

The retention of DA and NA normally and after PTP pretreatment

From Table I and II it appears that PTP does not reduce the ability of the caudate slices to retain amines (or catabolites). This is true regardless of whether DA or NA is used as a substrate. From Table I it appears as if PTP actually increases the amount of amines retained but this increase is not statistically significant.

The detailed catabolism of DA and NA

When the slices of the caudate nucleus are incubated with DA and each of the individual catabolites are isolated and quantitated it can be seen from Table III that the major catabolite is DOPAC with DOPET and HVA being formed in much smaller quantities. All of the other potential catabolites of this amine were formed only in negligible quantities when compared with the control values.

When the slices were incubated with NA, it can be seen from Table IV that DOPEG and DOMA are the major catabolic products with the other compounds formed in quantities which are either small or negligible.

Discussion

The results from this study indicate that the caudate nucleus, containing primarily DA neurons, differs in several metabolic properties from the cortex region containing primarily NA neurons. In agreement with the histochemical and biochemical data of Carlsson *et al.* (1966) PTP appears to inhibit the uptake of catecholamines in the NA neurons but not in the DA neurons. In the metabolic studies this inhibition of uptake into the neuron was reflected as a decrease in the amount of deaminated catabolites formed since the access of the amine to intraneuronal monoamine oxidase was reduced. This effect occurred in the cortex (Jonason and Rutledge 1968) but not in the caudate nucleus as observed in the present study. The effects on the catabolites were closely correlated with the amount of amine retained by the slices indicating that the reduction in deaminated catabolites was related to an inhibition of uptake of amines.

(DOPET) 11.5 % ± 1.4, 3-methoxy-4-hydroxyphenylethanol (MOPET) 16.6 % ± 1.2, noradrenaline (NA) 68.4 % ± 3.7, 3,4-dihydroxymandelic acid (DOMA) 14.8 % ± 1.8, vanilmandelic acid (VMA) 14.6 % ± 2.7, normetanephrine (NM) 68.4 % ± 1.7, 3,4-dihydroxyphenylglycol (DOPEG) 13.3 % ± 3.7 and 3-methoxy-4-hydroxyphenylglycol (MOPEG) 16.4 % ± 4.0. All values are corrected for recovery. For further explanation see Table I

MOPET	NA	DOMA	VMA	NM	DOPEG	MOPEG
3.6	18.7	13.0	31.8	5.1	22.0	13.0
1.0	2.2	3.6	1.8	0.5	—	2.3
3	4	3	3	3	1	2
0	18.5	0.5	4.0	10.5	1.7	0
0	1.7	0.5	2.7	—	1.7	0
2	2	2	3	1	3	2

TABLE IV Detailed catabolism of H NA in slices of rabbit caudate nuclei.
Slices of caudate nuclei were incubated with $5{,}760 \times 10^{-11}$ moles of H NA (5.27 or $7.12 \times 10^{-}$ curies). Mean recoveries of the metabolites were noradrenaline (NA) 65.4 % ± 2.1, 3,4-dihydroxymandelic acid (DOMA) 13.3 % ± 2.6, vanilmandelic acid (VMA) 14.7 % ± 4.6, normetanephrine (NM) 38.5 % ± 1.0, 3,4-dihydroxyphenylglycol (DOPEG) 11.8 % ± 1.7, 3-methoxy-4-hydroxyphenylglycol (MOPEG) 16.8 % ± 5.1. All values were corrected for recovery. For further explanation see Table I.

Treatment		NA	DOMA	VMA	NM	DOPEG	MOPEG
	Mean	3,940	206	14.0	185	239	37.0
Normal	S.E.M.	162	55	2.3	44	37	11.2
		3	3	3	2	2	3
	Mean	4,350	3.0	2.3	160	0.3	7.7
Control	S.E.M.	83	1.8	0.6	6	0.3	0.8
	n	2	2	2	2	2	2

The caudate nucleus also differs from the cortex in the negligible amounts of NA synthesized from DA. This is consistant with studies in which the endogenous levels of amines are found since the caudate nucleus is known to contain very large quantities of DA but smaller amounts of NA compared with other tissues (Bertler and Rosengren 1959, Bertler 1961). When the amines are injected intraventricularly the amount of NA synthesized in the rat striatum was negligible compared to that in the medulla and hypothalamus (Glowinski and Iversen 1966). It has also been shown that NA is formed in small quantities from tyrosine in bovine brain homogenates and tissue slices (Kindwall and Weiner 1966). This low activity in intact s or nonsupplemented homogenates could either be due to a lack of cofactors, deficiency of the enzyme or the presence of an inhibitory substance.

Another property concerning the metabolism of catecholamines in the caudate nucleus is that O-methylation appears to play a relatively minor role. It is interesting to note that even though the total amount of metabolism was found to be the same as in the cortex the amount present as O-methylated catabolites is relatively small in the caudate nucleus. When the slices were incubated with C DA the amount of MTA was 5 per cent of that in the cortex whereas the amount of the deaminated product, DOPAC was approximately the same (See Jonason and Rutledge 1968). The other O-methylated product, HVA, was present in quantities only 17 per cent of those in the cortex. When the slices were incubated with NA the two deaminated products, DOPEG and DOMA were present in quantities approximately equal to those in the cortex, while the three O-methylated products VMA, NM and MOPEG were found only in negligible quantities. This tendency has also been observed when NA was injected intraventricularly and the metabolites from each region were isolated (Glowinski and Iversen 1966). However when endogenous levels of the metabolites are isolated, considerable quantities of O-methylated compounds can be found

in the caudate nucleus as compared to other regions (Sharman 1963, Andén, Roos and Werdinius 1963, Carlsson and Waldeck 1964). The exact nature of this discrepancy cannot be explained at present.

The relationship between the metabolism of amines formed under *in vivo* conditions and the metabolism of exogenously added amines must await further knowledge of the relative rates of release, uptake into storage sites, uptake into the neuron, intra neuronal catabolism and extraneuronal catabolism under both the *in vitro* and *in vivo* situations.

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From the Institute of Neurophysiology University of Copenhagen, Denmark

The Excitability of Frog's Isolated Muscle Spindles during Repetitive Stretches

By

SAMIA AL AZHARIA JAHN

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Abstract

JAHN S AL AZHARIA. *The excitability of frog's isolated muscle spindles during repetitive stretches* Acta physiol. scand. 1968. 73 418—426.

In trains of stretches lasting 1 sec the minimum interval for response to all stretches (deformation 1—2 times threshold stretch (T) rise time 2 and 5 msec) was about twice the shortest interval for responses to a pair of stretches. When the trains lasted 3—6 sec and the stretches had the critical size only a few failed to evoke a response. The stretch which evoked a response to every stimulus of a train increased linearly with frequency (6—70/sec). At high frequencies, when adaptation had occurred, the rate of afferent discharges varied little with frequency the discharge rate being about half the highest obtainable and increasing linearly with the size of the stretch. Spontaneous intrafusal contractions facilitated the response to a train of stretches too small to evoke a response to every stimulus. The facilitation lasted as long as the contraction of an intrafusal muscle fibre. After a train of stretches (3 sec in duration) spontaneous afferent firing ceased. The silent period increased linearly with the number of afferent discharges independent of the frequency of stretches (35—70/sec) and of the size of the stretch (1.5—2.5 x T). The intervals between the spontaneous afferent discharges were prolonged for several seconds after a train.

When trains of supramaximal stimuli were applied to muscle fibres the number of successive responses decreased with increasing rate of stimulation. When the interval between stimuli equalled the absolutely refractory period, the third stimulus of a train failed to evoke a response (Farmer, Buchthal and Rosenfalck 1960). The aim of the study presented in this report was in a similar way to compare the effect of trains of stretches and of paired stretches on the excitability of the muscle spindle. In addition the changes in excitability of the muscle spindle were evaluated from the after-effect of a spontaneous intrafusal contraction on the response to a train of stretches and from the reduction in discharge frequency of the spontaneous afferent activity after a train.

Methods

Preparation. The experiments were performed on the isolated muscle spindles of m. extensor longus digiti IV of *Rana temporaria*. The preparation, the Ringer solution and the set-up to obtain transient stretches and to record intrafusally has been described (Jahn 1968 and

Preliminary reports Jahn (1963) and Jahn (1966)

1968 b) The initial elongation of the muscle spindles was 20—25 % and the temperature of the Ringer bath 17—20 C.

Stimulation and record g The rectangular pulses for the stretch device were obtained from stimulator which allowed single pulses to be repeated at arying intervals and to preset the duration of train (American Electronic 104 A)

Paired stimuli were repeated t rate of less than 1 per second. The rise time of the stretches was 2 or 5 msec, their duration 6 or 9 msec and the interval between the stretches of pair as aried from 8 to 60 msec. The duration of the train was 1—6 sec and the frequency of stretches in the train was aried in steps of 5/sec (6—20/sec) and of 10/sec (20—70 sec) The interval between trains was at least 5 min. The size of the stretches was expressed in units of the threshold (T) for an afferent response to t least 80 per cent of single stretches repeated at rate of less than 1/sec (Jahn 1968 b)

The silent period" of the spontaneous afferent firing after train of stretches was measured as the interval between the last evoked response and the first spontaneous action potential after the end of the train.

Results

A. Afferent responses to a train nd to paired st tches

1) *Str t hes f threshold size (T)* When the interval between stretches of a train was 35—50 msec, only the first few tretches evoked a response. These intervals were the shortest which alw ys evoked a response when paired stretches were applied (Fig 1 and Jahn 1968) When the interval was doubled (11—13 stretches/sec) all tretches of a train of 1—6 sec evoked a response (Fig. 2) Trains of 22—70 stretches/sec evoked about 15 afferent discharges/sec independent of the frequency of the train duration (1—6 sec, Fig 2 B and 4) and of th rise time of the stretches (and 5 msec)

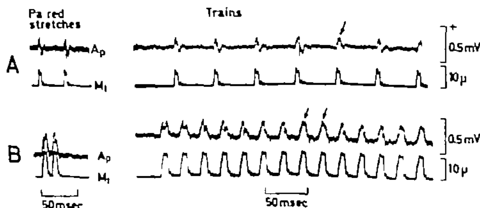


Fig. 1 Responses pa st hes and to rains.

A. Stretches of threshol l size B μ, rise time 2 msec) the shortest interval between stimuli of pair was 35 m When the inter al between stretches of train was 40 per cent longer (48 msec t. pindle failed to respond to the fifth stretch (arrow)

B Stretches of 1 imes threshold (11—12 μ, rise time: 5 msec) the shortest interval between stimuli of pai was 14 msec lef When the interval between stretches of train was 0 per nt long 4 msec) the spindle failed to respond to the eighth and ninth stretch (arrow)

Ap afferent pike superimposed on movement artefact.
Mt. mechanic l ements

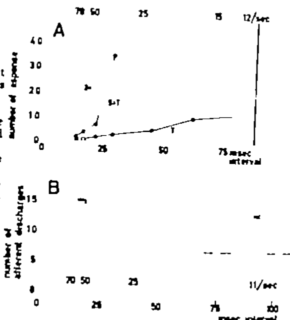


Fig. 2. Pattern of the afferent responses to a tral of stretches lasting 1 sec.
A: Number of subsequent responses in the beginning of the train with stretches of threshold size, 1.5×T and 2×T as function of the interval between the stretches.
B Number of afferent discharges (evoked responses and spontaneous discharges) during train of threshold stretches as a function of the interval between the stretches
The stippled line indicates the average rate of spontaneous afferent discharges.
●: rise time 2 msec.
× rise time 5 msec.

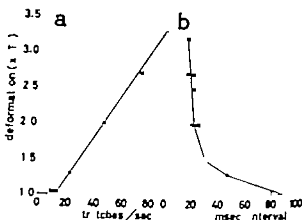


Fig. 3. Size of stretch (i units of T) which evoked responses to stretches during at least 1 sec the train as a function of the ( ) and of the interval stretches (b)
Rise times 2 msec × 5 msec.

2) *Suprathreshold stretches* When the stretch was two times threshold the spindle responded to trains (45/sec) with an interval of 22 msec At interval of 18 and 20 msec adaptation occurred. This minimum interval was also twice the shortest interval between the stretches of a pair which always evoked a response (size of the stretch 2×T Jahn 1968 c) The number of subsequent stimuli of a train which evoked a response increased linearly with the interval between the stretches and with the size of the stretch (Fig 1—3) When the interval was 25 msec or longer a small increase in stretch size enhanced the responsiveness of the spindle when the interval was below 25 msec, the sensitivity decreased progressively At low frequencies of stretch the duration of the trains did not affect the excitability of the spindle to suprathreshold stretches. The frequency at which all stretches just evoked a response when

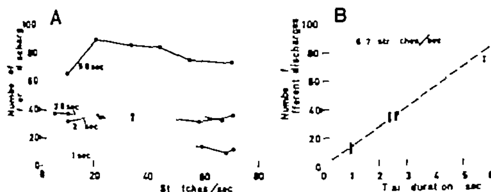


Fig. 4. Number of afferent discharges during stimulation with trains of stretches of threshold size (6—71 stretches/sec)
A. as function of the frequency (train duration 1—5.8 sec)
B as function of the duration of the train.

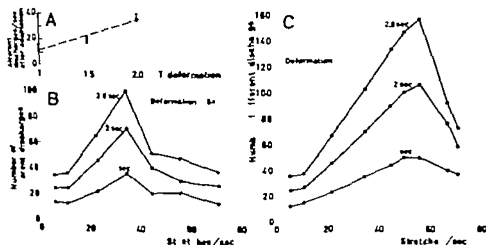


Fig. 5. The adaptation of the muscle spindle to stretches of suprathreshold size.
A. The steady rate of afferent discharges at frequencies of stretches failing to evoke response to every stimulus as function of the size of stretch.
B and C. The number of afferent discharges during stimulation with trains of suprathreshold stretches (1.5 and 2 x T) as function of the frequency of stretches. Note the decline of the discharge rate to "steady rate" at frequencies which failed to evoke a response to all stimuli. The trains lasted 1— 8 sec

the train lasted 1 sec caused 1—5 of the stretches to fail when the train duration as prolonged to 3—6 sec.

3) *The latency of responses* When the frequency of stretches was above that at which all stimuli evoked a response the latency of the last few responses before adaptation occurred was 1—3 msec longer than the latency of the response to the first stretch (Fig. 1) This is similar to findings with paired stretches when the interval between the stretches of a pair at threshold size was such that only one third or one half of the test stretches evoked a response (1 — 7 msec the la

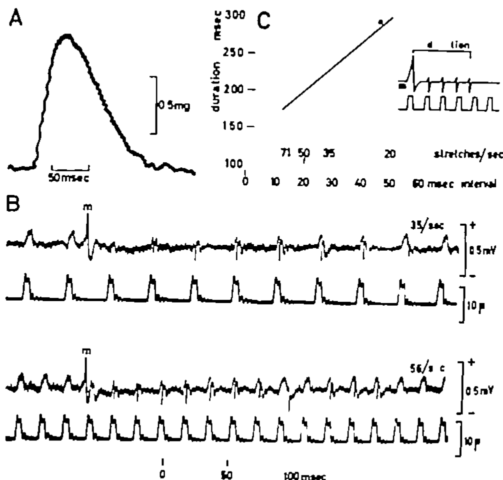


Fig. 6. Facilitation of responses by spontaneous intrafusal contraction.
A Intrafusal contraction.
B Facilitation of stretches during trains which failed to evoke response to every stimulus, m action potential of spontaneously contracting intrafusal muscle fibre.
C Duration of the facilitation as function of the interval between stretches.

of the response to the test stretch was 1—2 msec longer than that of the response to the conditioning stretch. When 80—100 per cent of the test stretches evoked a response (interval 35—50 msec) the latency remained unchanged.

4) *Evok d esponses and spontaneous afferent discha ges as related to the f e quency of t et h s St etche of th eshold si.e (T)* The highest rate of afferent responses evoked by trains of stretches lasting 1 sec was twice the rate at which spontaneous discharges occurred between the trains. When the frequency was 11—12 stretches/sec, 1—? spontaneous action potentials per sec occurred between the evoked responses. When the frequency of stretches was above 45/sec the discharge rate was about 15/sec (cf p 419)

Suprath eshold stretch s When the size of the stretches was 1.5—2.5 × T and the stretches were given at 11—.3/sec 1—? spontaneous action potentials occurred in

addition to the responses during the first second of the train. Additional afferent action potentials were fewer or did not occur at all when the trains were prolonged to 3—6 sec. When the size of the stretch was 1.5—2×T the frequency of afferent discharges evoked by rates too fast to elicit a response to every stretch changed only little with the frequency of stimulation and increased linearly with the size of the stretch (Fig. 5 A) This response frequency was about half the maximum frequency of afferent discharges as obtained when all stretches were responded to (Fig 5 B)

B. Th effect of spontaneous intrafusal cont actions on the response to a train of stretches

A spontaneous intrafusal contraction indicated by an action potential from an intra fusal muscle fibre (m" Fig. 6 B) facilitated the response to a train of stretches. During trains of threshold stretches an intrafusal contraction augmented the probability of response by 20—35 per cent. The facilitation of evoked responses was most evident at frequencies of 35—70/sec (Fig. 7) lasting as long as the intrafusal contraction (Fig. 6) The time interval in which all stimuli of the train evoked a response decreased with increasing frequency of stretches (Fig. 6 B and C)

Trains of stretches below threshold size (0.5×T) failed to summate. However when a spontaneous intrafusal contraction occurred during trains of 11—35 stretches/sec two to five subsequent stimuli evoked a response at higher rates no facilitation was observed. When the trains of stretches were above threshold (1.5—3×T) facilitation by an intrafusal contraction could be detected only when the size of the stretch was too small to evoke a response to every stimulus. At low frequencies of

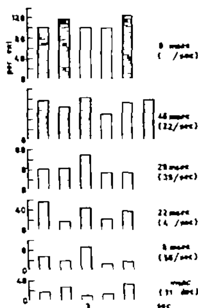


Fig. 7 The effect of spontaneous intrafusal contractions on the rate of afferent discharges during stimulation ith trains of stretches of threshold size (T) The figures to the right denote the intervals between stretches and the columns indicate the percentage of stretches hich evoked response (or was accompanied by spontaneous afferent discharge) during consecutive seconds of the train (abscissa) The hatched columns denote the seconds in which spontaneous intrafusal contraction occurred, indicated by the occurrence of muscle action potential

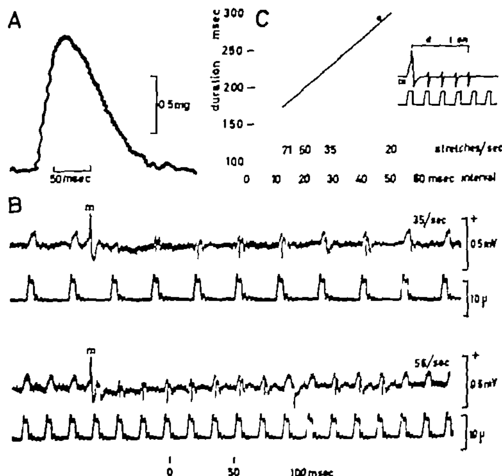


Fig. 6. Facilitation of responses by spontaneous intrafusal contraction.
A. Intrafusal contraction.
B. Facilitation of stretches during trains which failed to evoke response to every stimulus, action potential of a spontaneously contracting intrafusal muscle fibre.
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of the response to the test stretch was 1—2 msec longer than that of the response to the conditioning stretch. When 80—100 per cent of the test stretches evoked a response (interval 35—50 msec) the latency remained unchanged.

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Suprathreshold stretches When the size of the stretches was 1.—.5 × T and the stretches were given at 11—23 sec, 1—? pontaneous action potentials occurred in

6/sec for about two sec after the train (Fig. 8) even 15 min after the train the rate of spontaneous discharges was 1—2/sec lower than at the onset of the experiment. Repeated trains delivered at intervals of 5—10 min did not decrease this rate further.

Discussion

The recovery of the excitability of the muscle spindle as it appeared in the responses to trains of stretches lasted 50—100 per cent longer than when tested with paired stretches. This difference could be explained by a progressive (cumulative) increase of the latency of the responses preceding the failing of the afferent response to the stretches of a train (Fig. 1) This is due to a progressive shortening of the interval between each response and the following stretch.

The adaptation of the muscle spindle above frequencies at which every stretch evoked a response occurred at a rate which was about half the maximum obtained when all stretches of a train evoked a response. This lower rate of action potentials at a high frequency of suprathreshold stretches may be caused by the visco-elastic properties of the tissue surrounding the sensory terminal reducing its deformation at the high repetition rates.

The fluctuation in the number of afferent action potentials evoked by stretches smaller than those which evoked a response to every stimulus was due to a facilitation caused by an intrafusal contraction. Driving" of the afferent discharges by repetitive motor nerve stimulation occurred in muscle spindles of the frog at rates below 20/sec (Matthews and Westbury 1965) and in the toad at 10—12/sec (Eyzaguirre 1958) Contractions of single intrafusal muscle fibres, which cause a stretch of the equatorial region of 1—1.5 × T (Jahn 1966) can be expected to evoke afferent responses at a driving rate" of 10—20/sec (Fig. 3)

A similar post-stimulation depression of the spontaneous afferent firing of muscle spindles as in the frog has been described in the mammalian muscle spindle after stimulation of γ-fibres (Kuffler Hunt and Quilliam 1951) and in frog muscle spindles after release from stretch (Matthews 1931 Katz 1950) Also in Pacinian corpuscles the recovery time to stimuli of threshold size increased with the number of stimuli after a prolonged mechanical stimulation (Loewenstein and Cohen 1959) the delay of recovery depending in addition on the strength of the preceding stimuli. In muscle spindles an increase in the size of stretch from 1.5—2.5 T did not increase the silent period of the spontaneous afferent firing after a train. Prolonged repetitive stimulation at an interval which allowed f[illegible]ll recovery when applied as paired stimuli (30—40 msec, Jah[illegible] 1[illegible]) depressed [illegible] spont[illegible] afferent firing. This, indicates a "latent fat[illegible] [illegible]hich ma[illegible] be a f[illegible]rther reason for the difference in the recovery of excitab[illegible] [illegible] and after repet[illegible] [illegible]tretches. The post-stimulation depression [illegible] discharges wa[illegible] [illegible] when the average interval between action p[illegible] 70 msec [illegible] duration 6 sec. Thus, the low frequencies at which [illegible] [illegible]red [illegible] muscle spindles can hardly initiate such fatigue

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Histochemical Demonstration of Carbonic Anhydrase Activity in Some Epithelia Noted for Active Transport

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Abstract

HANSSON H. P J *Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport.* Acta physiol. scand. 1968. 73 427—434

An improved histochemical method for demonstration of carbonic anhydrase has been applied to a number of epithelia engaged in electrolyte transport.

Heavy staining was found close to or in the following regions.

1 Intercellular spaces bordered with folded epithelial membranes (unpigmented epithelium of rabbit ciliary processes, straight segment of human eccrine sweat gland duct, rabbit gall-bladder and villi of rat duodenum)
2. Basal membrane infoldings (rat proximal and distal tubules, pigmented epithelium of rabbit ciliary processes)
3. Intracellular canaliculi (parietal cells of rat stomach)
4. Brush border of rat proximal tubule.
5. Certain nuclei, but only in fixed sections and of dubious specificity

Moderate to weak staining was seen in several other places in the epithelial layers.

It is well known that carbonic anhydrase (carbonate hydro-lyase E.C. 4.2.1.1) activity can be demonstrated in a variety of tissues known to transport electrolytes. The importance of carbonic anhydrase activity for secretory function has been studied after inhibition of the enzyme by specific sulfonamide-type carbonic anhydrase inhibitors (Review Maren 1967) By the aid of histochemical methods (Häusler 1958, Waldeyer and Häusler 1959) a growing body of information has been collected on the presence of carbonic anhydrase in secretory epithelia such as the kidney tubule (Häusler 1958) the mucosal epithelium of the digestive tract (Korhonen, Korhonen and Hyyppä 1966) and the epithelium of the ciliary body (Korhonen and Korhonen 1965 Leder 1966

In some epithelia the staining reaction performed even with the conventional technique suggests the presence of carbonic anhydrase where l ng narrow membrane-bordered channels are found in electron microscopy th t ellular canaliculi of the parietal cells of the rat stoma h (Vollrath 1959) and the basal membrane

infoldings of the striated duct of the rat parotid gland and distal tubule of the rat kidney (Leder and Tritschler 1966). The author has recently described an improved histochemical method (Hansson 1967) for the staining of carbonic anhydrase. It is a modification of the classical method of Häusler (1958) and permits better resolution by the use of thinner sections and much shorter incubation time.

The method has now been applied to a number of epithelia which are known from electron microscopy to have extra-cellular channels of three types: lateral intercellular spaces, basal membrane infoldings and intracellular canaliculi.

Material and methods

Rabbit tissue samples (anterior part of the eye and gall-bladder) wer[illegible] taken immediately after the animal was killed by intra[illegible]venous ai[illegible]. The samples wer[illegible] cut down t[illegible] maximal thickness of 1—2 mm and quenched in isopentane cooled with liquid nitrogen.

Punch biopsies (3 mm diameter) were taken from human ski[illegible] without anesthesia. They were immediately frozen in isopentane-filled tubes which had been [illegible]hilled to −70 °C in ethyl alcohol and dry ice.

Rats were perfused [illegible] vivo under ether anesthesia with 6.25 % glutaraldehyde in 0.15 M cacodylate buffer [illegible]t pH 7.3. The perfusion was made through the abdominal aorta and lasted for 10 min. At the start the left renal [illegible]ein was cut open to secure free flow of fixati[illegible]. The tissues removed (kidney, stomach and duodenum) were trimmed to 1—[illegible] mm size and fixed [illegible]t +4 °C in the perfusion sol[illegible]tion for 20 hrs. They were then quenched and sectioned as [illegible]bbit tissues. I[illegible] some experiments slices of fresh frozen rat kidney w[illegible]re also used. These animals were killed by ether.

The frozen tissues were mounted on [illegible] cryostat chuck according to the method described by Pearse (1961). Sections w[illegible]re cut 5—7 μ, from the ciliary epithelium 10 μ thick, in a Pearse-Slee [illegible]ryostat [illegible]t −20 °C. Those from the rabbits and human skin were transferred on dry ice to a Pearse "Speedivac" Tissue Dryer (Edwards High Vacuum Ltd., Crawly, England). Freeze-drying was done in ac[illegible]ordance to the instructions, [illegible] 2—3 min. The sections from the rat, however, were thawed on [illegible] Millipore filter (25 μ thick, pore size 0.45μ) (Millipore Filter Corporation, Bedford, Mass., USA). This was found to preserv[illegible] the histological feature[illegible] and simplified the subsequent procedures.

Histochemical proc[illegible]d[illegible] for [illegible]arbonic [illegible]nh[illegible]d[illegible]ase. The method has been described in detail [illegible]where (Hansson 1967). Sections are floated on the surface of [illegible] freshly mixed incubation [illegible]um in [illegible] 9.5 [illegible] in Petri dish. The medium is prepared as follow[illegible]: T[illegible] solution contains [illegible]ml 0.1 M $CoSO_4$, 6 ml 0.5 M H_2SO_4, 1—10 ml 1/15 M KH_2PO_4 and distilled w[illegible]ter to 17 ml is added [illegible] freshly prepared sol[illegible]tion of 0.75 g $NaHCO_3$ in 40 ml distilled water. [illegible]hich gives an excess of dissolved CO_2 [illegible] the final pH 5.8. If [illegible] Millipore support is used the sections must be on the top of the floating filter. Carbon dioxide is blow[illegible] over the surface for 10 min (by means of an inverted f[illegible]unel placed over the Petri dish) to allow the [illegible]arious ions to equilibrate in the sec[illegible]ions. Wh[illegible] the carbon dioxide atmosphere is removed a cobalt-phosphorous [illegible]ompound precipitates [illegible]t sites of carbonic anhydrase activity. The addition of more phosphate to the medium makes the method more sensitiv[illegible] and the amount [illegible] is chosen so as to giv[illegible] the staining reac[illegible]ion [illegible]fter as short an [illegible]ncubation as possible, usually 3—10 min. The amount of KH_2PO_4 [illegible]nd [illegible]he [illegible]ncubation time are show[illegible] under each figure. The [illegible] [illegible]ions to the surfac[illegible] [illegible] [illegible]tical and [illegible]t is thus essential to mak[illegible] sure that the sections do not drop unde[illegible] the surfac[illegible] of [illegible]he m[illegible] [illegible]. If [illegible]hey do [illegible]hey [illegible] remain unstained. The sectio[illegible] are rinsed for 3 min in physiological saline buffered [illegible]ith phosphat[illegible] ($6.7 \times 10^{-}$ M, pH 5.9). The precipitate is made [illegible]isible [illegible] (CoS) [illegible] 0.5 % $(NH_4)_2S$ in distilled [illegible]ater and the sectio[illegible] rinsed for 1 min in [illegible]ach of [illegible] [illegible] [illegible] saline dishes. Sections are mounted [illegible] [illegible] jelly or dehydrated through [illegible] [illegible]oncentrations of ethanol [illegible]nd xylene and mounted [illegible] Canada balsam. Sections on [illegible]he Millipore f[illegible]lter [illegible] dehydrated through [illegible] ethanol, absolute n-propanol and xylen[illegible] [illegible] [illegible]lene [illegible]nd mounted in Canada balsam. For some of the sections [illegible]ounterstaining was perf[illegible]rmed with toluidine blue or haematoxyline and eosin. Photomicrographs [illegible]ere taken on P[illegible] [illegible] Kodak film, using a ×100 Zeiss oil immersion lens, N.A.=1.25 and [illegible] Zeiss f[illegible]lter [illegible] [illegible] mm [illegible]hick. [illegible] [illegible]he figures the [illegible] represents 10 μ.

Control Experiments

1. The inhibitor sodium acetazolamide (10^{-4} M) is included in the medium. 2. An atmosphere of CO_2 is kept above the solution with the floating sections. This prevents the escape of CO_2 which precipitates the cobalt-phosphorous compound and is the basis of the reaction. 3. Sections on Millipore filter are incubated in medium lacking the substrate but in such manner that cobaltous compounds are precipitating for non-enzymatic reasons. As described in detail elsewhere (Hansson 1967) the medium is composed of 10 ml 0.1 M $CoSO_4$, 3.75 ml 0.8 M Na_2SO_4, 10 ml 1/15 M KH_2PO_4 and 32.5 ml distilled water. A drop of 0.05 M barbitalbuffer pH 9.1 is placed on the free surface of the sections. Cobaltous compounds then commonly precipitate in the sections where the two solutions meet and neutralize each other.

Results

Sites for staining reaction

Lateral intercellular space (Figs. 1—4) In the ciliary epithelium of albino rabbits (Fig. 1) the heaviest staining is between the apical parts of the non-pigmented cells. The stained region forms a wedge with its base facing the posterior chamber. There is also some staining, but much less, at the basal surface of the pigmented cells facing the stroma of the ciliary processes. The outline of all the cells is faintly stained. *It is still uncertain if there is any part of the apical surface of the non-pigmented cells totally free from staining.*

The pattern of staining at the lateral border of the cells is seen also in the luminal cells of the straight segment of the human eccrine sweat gland. (Fig. 2.)

The epithelium of the rabbit gall-bladder exhibits most of the reaction product

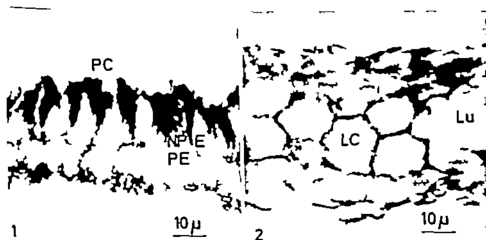


Fig. 1. Ciliary process epithelium (unfixed) of albino rabbit eye. Most activity is found at the lateral cell borders of the non-pigmented epithelium (NPE) and at the basal parts of the pigmented epithelium (PE). PC = posterior chamber. 10 ml 1/15 M KH_2PO_4 in the medium. Incubation time 6 min. Lightly counterstained with haematoxyline and eosin.

Fig. 2. Human eccrine sweat gland, straight segment (unfixed). Cross section of hexagonal luminal cells (LC) with the activity shown at the lateral cell borders. The staining ... the cell apex at the lumen (Lu). 6 ml 1/15 M KH_2PO_4 in the medium. Incubation time 6 min. Lightly counterstained in toluidine blue.

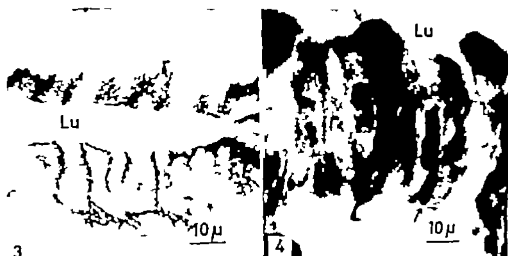


Fig 3 Rabbit gall-bladde pithelial crypt (unfixed) The staining reaction is mainly found t the la eral cell borders and the cell base I the longitudinally sectioned ells, the apices in the pper part of the crypt (to the left) show no activ t whereas the low ones to the right show inten staining 10 ml 1/15 M KH_2PO in the medium. I cubation time 5 min. No ounterstain

F g 4 Ra duodenum epithelium at top of a illus (fixed) Lu=lumen The arrow point to wedge like deposits between the cells. The pical staining is not the brush border 10 ml 1 15 M KH PO the medium Incubation time 10 min No counterstain

adjacent to th lateral cell borders, onl smaller amounts basally (Fig 3) The epithelium of th deep parts of the folds (to the right in the figure) showed a staining t the luminal border which was otherwise absent or ery parse (to the left) Throughout the cytoplasm, especially in the supranuclear part a grainy pre e was seen mitochondria) The epithelial cells at the base of the llus of rabbit duodenum showed no reaction. From the middle to the t p of the villus asing mount of reaction products were found. In the epithelial cells w th the least acti t only a faint reaction was found at the lateral borders of th basal half of the cells. The nuclei we unstained Nearer the top a complex sta g pattern of the cells appeared (F g 4 The who e of the lat ral cell borders w re outlined w th bl k deposits, which f rmed wedge-like patterns with the thick end pically and or ba all arrow Th r was no taining of the striated border b t bene th it, a moderately stained longitudinally striated zone was found the longitudinal striation is not sibl n the print On the apical side of the nucleus nd to a smaller extent n the basal side a grainy precipitate is seen (mitoch ndria) The nuclei of the cells exhibit ng thi entire taining reaction were also int n el stained *B cl memb a f ld F g 5-7* The proximal and distal t bules in the r t kidney cortex F gs 5- 7 z the same taining reaction whether the sections were fresh frozen or fixed n gl tara deh de There was one exception th nu lei f the proximal tubules but n t those f the di tal ones were stained wh fixed sec tions were used. This marked taining of the nuclei and nucleoli c n be complete) inhibited with acetazolamide but ma still be non- pecific (see bel w The sma

distributions are described by Häusler (1958) With the present method, however more details appear In the proximal tubule (Fig 5) the hea est staining occu red in a narrow basal zone. Another zone of strong staining is the luminal surface Ther is no clear-cut accumulation at the lateral borders. As shown in Fig 6 precipitation has also occurred outlining what seems to be basal plasma membrane infoldings (arrow) The brush border too shows up. In the distal t bule (Fig. 7) the staining reaction was restricted to the basal part of the cells where a markedly striated pre cipitation pattern appeared. The erythrocytes show variable staining in these fixed sections. It is remarkable that in the distal tubule the staining reaction in the basal parts of the cell where infolded basal plasma membranes are located close to the elongated mitochondria never took the shape of membrane infoldings found in adjacent proximal tubules. It is possible that the striated staining seen in the distal cells is due to something else than the folds of the membrane, perhaps the elongated mitochondria.

Intracellular canaliculi (Fig 8) The parietal cells of the rat gastric gland showed an intense staining reaction, more in the lower part of the gland, less at the neck In the cytoplasm the precipitate forms a net-like pattern, spottily accentuated at the cell borders. In some cells, the staining reaction revealed an elongated termination of the cell apex at the glandular lumen (Fig 8) Some nuclear taining was also found. Apart from the nuclear staining this distribution is in accordance w th the findings of Vollrath (1959) who interpreted the net like pattern as due to staining of the intracellular canaliculi.

Specificity tests

In the control experiments of type 1 and 2 no staining was present after incubation in the presence of inhibitor (10^{-4} M acetazolamide) or when CO_2 was blown over the surface of the medium and thus the reaction was specific by these criteria

As already pointed out (Hansson 1967) staining of the nuclei or nucleoli may be non-enzymatic. Since these sites of staining were found in the kidney duodenum and gastric mucosa of the rat, the localization of a non-enzymatic precipitate was studied in sections from these organs.

In control experiments type 3 the distribution of staining was diffuse and showed no preferential localization except to nuclei and nucleoli. These then must be re garded as sites of non-enzymatic staining until th opposite has been proved.

In other experiments sections were incubated on th surface of a medium containing acetazolamide in high concentrations (1 75 x 10 M) but for such long periods that the uncatalyzed dehydration of H_2CO_3 caused precipitation at the surface of the solution. The reaction product could not be related to any cellular structures.

When the enzyme activity is partially nl bited by acetazolamide the time for th precipitation threshold to be reached is nc eased Th conditions for the appearence of false positive reaction sites du to diff ion of the reaction products still in solution would then become more favourabl In uch experiments when sections from

In this connection the possibility of some loss of cytoplasmatic soluble carbonic anhydrase must be remembered. It is conceivable that such a loss occurs from the sections and causes undue predominance of the enzyme localized close to membranes and presumably bound to structures in the membrane region.

Thus, with the exception of nuclei and nucleoli, the sites of staining reactions evidently seem to be sites of enzyme activity even at the subcellular level at least as far as major regions of cells are concerned, but no claims can be made for what the electron microscope might show

Enns (1966) has recently pointed out that carbonic anhydrase might play a part in the penetration of CO_2 through biological membranes. It is possible that at least part of the apparently membrane localized enzyme has such a function.

It should finally be mentioned that the ATPase demonstrable with the Wachstein-Meisel medium is localized in the same regions as those in which heavy staining for carbonic anhydrase was found in the present study (For references and a criticism on the interpretation of these findings, see Tormey 1966.)

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Beta Adrenergic Blockade and Central Circulation during Exercise in Sitting Position in Healthy Subjects

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Abstract

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The effect of beta-adrenergic blockade on central circulation during exercise in sitting position has been examined in 8 healthy young men. After oral administration of propranolol in doses of about 0.22 mg/kg b.w.t. there were significant decreases in pulse rate, cardiac output and mean pressure in the brachial artery during exercise. An increase of a-v oxygen difference and of mean pressure in the pulmonary artery was noted. No systematic change was noted in oxygen uptake and stroke volume respectively during the beta-blockade. The physical working capacity at pulse 170 increased on an average by 15 % after propranolol. This increase was made possible primarily through better peripheral oxygen utilization and could only be explained to minor extent by an increase in stroke volume.

It has been shown in previous studies that the physical working capacity at pulse 170 (W 170) increases during a beta adrenergic blockade with propranolol. Furthermore closer relationships were brought about between W and the circulatory dimensions, heart volume and the total amount of hemoglobin after propranolol in a group of patients with various psychiatric diseases (Furberg 1967a). The effect of the blockade on W 170 was on an average higher in patients with signs of a hyperkinetic circulation than in subjects with signs of normo- or hypokinetic circulation (Furberg 1967b).

Results from hemodynamic studies on the effect of propranolol on healthy subjects during heavy work have been published over the last few years (Epstein *et al.* 1965, Cumming and Carr 1966). In these investigations the subjects worked on a treadmill or in a supine position using a bicycle ergometer. Substantial differences in the hemodynamics, cardiac output and oxygen difference has been shown to exist in healthy subjects between exercise in supine and sitting positions (Bevegård, Holmgren and Jonsson 1960). It is known that the blocking effect of propranolol on different responses is dependent on the dose (Gebhardt *et al.* 1965, Ablad *et al.*

1967) In the related hemodynamic studies higher doses of propranolol were used than in Furberg's previous work (1967a and b)

This hemodynamic study was made to elucidate the previously reported effects of propranolol on W_{17} recorded during heavy exercise in a sitting position.

Material

The material consisted of eight healthy male students. Their physical acti ity was ordinary none at the time took part in athletics. Prior to the atheterization they underwent an examination including linical history auscultation of heart nd lungs, hest X ray and ECGs t rest and during work. None of them showed any signs of somatic disease. Some anthropometric data on the subjects appears in Table I

Methods

Prior t the investigation the subjects wer informed of the methods and the aim of the study Determinations of the physical working capacity heart olume (HV) and the total amount of hemoglobi (THb) w re made within one week before the catheterization.

An electrically braked bic le ergometer (Holmgren and M ttsson 1954) as used to determine the *physical wo k g c pacity at pulse at 170 beats/m* (W 70) according to Sjöstrand (1947) and Wahlund (1948) The subjects started working t work load of 300 kpm/min and the load was the increased stepwise ev ry 6th min by 300 kpm/m until a pulse rat of about 170 was obtained. By inter or extrapolation, assuming linear elationship between pulse rat and work load, the work intensity i kpm/mi at pulse rat 170 as obtained. During the tests all subjects w re i relatively steady stat i.e the pulse rat did not increase more tha 10 beats from the second to the sixth min of work or not more than 3 beats between the fourth and sixth minute. The heart rate was recorded t rest and after standing for 8 min befor the work.

H rt ol m (HV) was det rmined in the prone position by the modified Larsson—Kjellberg method (1948) with the central ray of the frontal picture t an angle of 30 caudally to the ertical and w th distance of 1.5 cm between the focus and the film. The heart olume was calculated in principle with Jonsell' formula (1939) according to a me hod sed by Linderholm and Strandell (1958)

Total mount f hemoglobin (THb) was determined according to the al eola CO method with slight modification of the original method (Sjöstrand 1948, Holmgre *t al* 1957) Smaller amounts of CO w added to the rebreathing system and the CO anal se ere made according to Anderson and Dahlstrom (1958) The blood olume was alc la ed from the Hb and the hemoglobin concentration in finger blood

ght heart athet izatio was arried out the usual manner N seda es ere given to the study The subjec were allowed to hav slight meal 2 hrs befor the examination started T mak the nsertion pa nless sufficient local anesthetics w used A teflon atheter was in roduced int the brachial rtery sing the percutaneous techniqu (Seldinger 1953 A double-lumen heart atheter was placed with its tip in the left or right pulmonary artery Lef to righ and righ to left intracardiac shunts of significanc er excluded.

Blood pr ssu w recorded on an Elema Klinik ECG apparatus using the Elema strain gauge mechano-electrical ransducer The insertion of the fou th ib t the ter num as taken as the zero pressu level d ring the work tests in sitting position. Dur the ardiac catheterization the subjects performed work loads. The second load was hosen to gi pulse rate of abou 1 0 be ts mi The f rst load w half the second one

Cardiac output was determined a ording t the direct Fick principle *O y n* *ptake* as measured using he Dou las ba echnique During exer se the ollection f expired air as made bet een the fourth and seven h min of exercise on ach work load Samples of arterial and mixed enous blood were d off simultaneousl during the collection f expired air

Gas nd blood analyse The micro-Scholander method 1947 was sed for the determination of O_2 and CO_2 of the exp ed ai The rror of single determination has been alculated f om d plicate determina f O_2 and CO_2 and was expressed oefficients of ariation, 0.2 and 1.0 r respectiv l The expired gas olume was measured th g someter The orresponding error of sin le det rmination of gas olume as 0 The O_2 content and O_2 saturation of blood as measured by means of pectrophotometric method (Holmgren and Pernow 1959 The hemoglob oncent tion was me sured as oxyhemoglobin

(Sunderman *et al.* 1953) The error of single determination of O_2-saturation (20—65 %) and Hb-concentration (more than 10 g%) has been calculated from duplicate determinations and includes errors of sampling and analyses. The coefficients of variation were 1.0 % and 1.1 % respectively.

After the insertion of the catheters the subjects rested 30 min to reach a condition as basal as possible. About 15 min after completion of the first work test in sitting position propranolol was given in an oral dose stated below. Sixty min later a second work test was performed in the same manner as the first one.

Cardiac output at rest was determined in some subjects. One of these cases experienced sudden imminent syncope. His symptoms rapidly disappeared when the exercise started. As our main purpose was hemodynamic studies during work, we did not want to run the risk of syncope in the following catheterizations and thus we gave up the examinations at rest.

The dosage of propranolol (Inderal, ICI, England) was determined by body weight. Fifteen mg was given orally to subjects weighing between 60 and 75 kg and 20 mg to the one exceeding 75 kg. Conventional statistical methods were used for calculating the arithmetic mean and the standard deviation. The significance of differences between mean values was tested by Student's t-test (Snedecor 1956). The Spearman rank correlation coefficient was used for testing the significant limits of correlation (Siegel 1956).

Results

Data obtained before the catheterization

The mean of the *heart volume* (HV) in the supine position was 758 ml (Table I). All subjects had an ordinary heart volume in relation to the total amount of hemoglobin.

The total amount of hemoglobin (THb) was on an average 733 g, which was slightly lower than the amount estimated from body weight (11 g/kg b. wt.). The *hemoglobin concentration* was lower than 13.0 g per 100 ml blood in two subjects who were blood donors (Table I).

The mean *pulse rate at rest* was 71 beats/min while the corresponding rate *after 8 min standing* was 87 beats/min (Table I).

TABLE I. Some anthropometric data on 8 young healthy subjects. 0 means values before and I during beta-adrenergic blockade.

Case no.	Age, yr	Height, cm	Weight, kg	Heart vol. (supine), ml	Total amount of Hb, g	Hb conc. g/100 ml	Pulse rate (rest) beats/min	Pulse rate (standing) beats/min	Work intensity kpm/min at pulse rate 170 beats/min: Before cath.	During cath. 0	During cath. I
1	24	180	72	640	630	11.8	78	78	1 210	1 130	1 240
2	22	189	74	890	790	13.4	68	87	1 010	1 020	1 060
3	20	176	67	615	640	13.1	98	98	700	670	820
4	22	188	65	730	700	12.1	69	90	1 260	930	1 1[illegible]0
5	21	186	68	800	730	13.8	65	93	990	980	1 190
6	24	180	73	[illegible]00	790	14.4	75	85	1 000	870	1 150
7	21	174	66	790	700	11.0	64	88	960	1 040	1 100
8	22	182	75	900	880	13.2	51	71	1 500	1 510	1 600
M	22	182	71	758	733	13.1	71	87	1 079	1 019	1 166

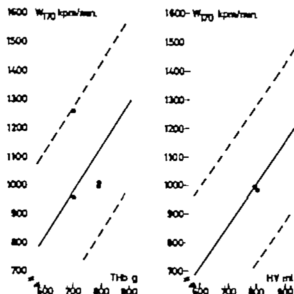


Fig. 1 Physical working pacity at pulse 170 (W 1) in relation to heart olume (HV) and the total amount of hemoglobi (THb) The regression equations are W 170=1 H HV – 170 S_e – 148 and W_{170}= 1 60 THb – 141 S_e – 150 (Holmgren et L 1959)

The mean *ph ysi al working capacity at pulse 170 be ts min* (W_{170}) in sitting position before the catheterization was 1079 kpm/min (Table I) W_{17} was in 5 sub jects lower than the W_{170}-values calculated from heart volume and the total amount of hemoglobin (Holmgren *et al* 1959) Three cases had a somewhat high W_{170} in relation to their circulatory dimensions (Fig 1)

Data f om the hea t atheterizations b f re and during beta-adrenergic blockade The mean values of th *puls rat* during work were 120 and 174 beat min before the blockade Aft r propranolol was given there was a significant decrease in heart rate to 114 and 160 respecti ely The decrease in pulse rat at the second load was about 8 (Tab II Fig 2)

No systematic difference wer noted in *oxyg n ptak* between values before and during the blo kade The mean oxygen ptake was 1 19 l min at the first and 2 17 n at the sec nd w k load before the blockade (Table II)

Th *, ct at f mi d ou blood* decreased on the first load from 43 7 t 38 7 d ring the blockade while the corresponding mean dec ase on the second load w fr 31 4 t 28 1 These changes re highly signif nt (Table II)

The related decrease gen sat ration in mixed v nous blood d ring the blockade brought t an in re se in the mean *a t ve us yg d ff r n* It increased on a ge by 7.8 ml l t 104 ml l on the first load and by 6.7 ml l to 127 0 ml l on the second Th l tter ncre se was f magnit de of bout 5.5 (Table II Fig

During as compared t bef th b ockade there were small b t significant changes in the mean *diac tp t* n b th loads The decrease fr m 18 1 before the blockade on the second load t 17 3 l min after t correspond to a perc ntal decrease of 4 (T bl II

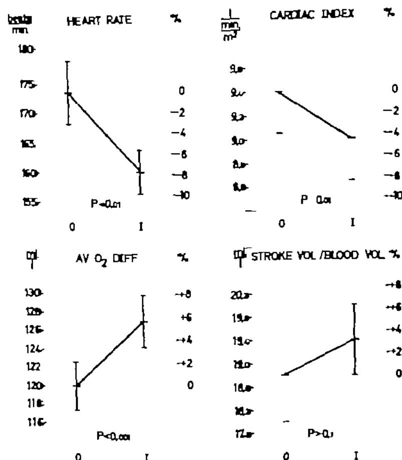


Fig. 2. The effects of propranolol on 4 circulatory variables during heavy work in 8 young healthy subjects. Mean values (±standard error of the mean) are shown for each variable. O means values before, and I during beta-adrenergic blockade. The scales on the right show percental changes of the mean values between the examination before and after propranolol. P = level of significance.

The *stroke volume* before the blockade was on an average 104 and 106 ml during the first and second load respectively. The corresponding mean values during the blockade were 100 and 109 ml respectively. The mean stroke volume and the mean quotient *stroke volume/blood volume* were 3 [illegible] higher at the second load during the blockade. This change is not significant (Table II, Fig. 2).

The *mean pressures in the pulmonary artery* before the blockade were on an average 19 and 25 mm Hg. The individual values were similar to those reported by Bevegård, Holmgren and Jonsson (1960). After the blockade the mean pressures increased significantly to 22 and 28 mm Hg respectively (Table II).

The *mean pressures in the brachial artery* during the blockade on the contrary decreased significantly on both loads from 99 and 110 mm Hg before the blockade

TABLE II Data obtained during right heart catheterization in 8 young healthy subjects. 0 means values before and I during beta-adrenergic blockade.

Case		Pulse rate, beats/min		O_2-uptake, l/min		O -satur in mixed en. blood,	
		0	I	0	I	0	I
1	Load I	122	116	1.29	1.24	42.0	32.5
	Load II	176	167	2.38	2.44	26.4	22.0
2	Load I	119	112	1.20	1.16	46.8	42.9
	Load II	168	164	2.17	2.14	36.3	33.9
3	Load I	143	131	1.03	1.10	46.6	42.6
	Load II	198	178	1.85	1.80	35.6	31.8
4	Load I	124	114	1.11	1.10	47.6	40.0
	Load II	177	156	2.01	1.98	33.3	30.1
5	Load I	111	107	1.09	1.13	44.3	38.0
	Load II	172	153	1.99	2.06	[illegible]	28.2
6	Load I	128	118	1.18	1.18	43.1	39.2
	Load II	185	158	2.13	2.21	29.2	25.7
7	Load I	108	115	1.17	1.18	41.7	39.8
	Load II	163	161	2.13	2.25	32.8	28.1
8	Load I	103	102	1.42	1.41	37.6	34.6
	Load II	147	143	2.69	2.69	29.1	24.7
	M	120	114	1.19	1.19	43.7	38.7
Load	I diff (0 I)	−6		_0		+5.0	
	P	0,05				0,001	
	M	174	160	2,17	2,20	31.9	28,1
Load II diff	0 I	−14		−0.03		3.8	
	P	0,01		0,2		0.001	

7

to 91 and 104 mm Hg after it (Table II). The indi idual val es wer n relation to pulse r tes f the me magnitude as those found by Bevegård, H lmg n and Jonsson 1960

Effects of p op d l ll d ng th ath terization

The mean value f W bef re the blockad was 1019 kpm min. It increased after pr pranolol to 1166 kp min Table I The individual changes in W r d ring the adrenergic blockade ha been orrelated to some ariables that r influenced by variations in the a tonom t n Ther was a probable significant corr lation between an increase n W d ring the blockade and a high pulse ate at rest bef e the catheterization P<0 0 , N orrelation was noted when the increase in W170 during the blockade was relat d t th pulse rate after 8 min standing before the catheterization or to the deviat n f W f m regression lines W_{17} —HW and

A—V O_2 diff., ml/l		Cardiac output, l/min		Stroke volume, ml		Mean pressures, mm Hg PA		BrA	
0	I	0	I	0	I	0	I	0	I
93	104	14,0	11,9	114	103	20	24	99	93
125	127	19 1	19,2	109	115	25	29	114	111
90	94	13 4	12,3	113	110	16	19	95	93
114	118	19 1	18,2	114	111	24	26	110	101
[illegible]	100	12,2	11 1	85	84	21	26	120	116
113	120	16,3	15,0	82	84	27	33	125	122
[illegible]	100	12,4	11 0	100	97	19	20	88	80
114	121	17 7	16,4	100	105	—	25	92	86
101	109	10,8	10,4	97	97	17	19	99	91
123	133	16,2	15,5	94	101	20	23	105	101
109	112	10,9	10,6	85	90	18	20	101	77
133	143	15,8	15,5	88	[illegible]	22	28	114	101
102	106	11,5	11 1	106	96	20	22	[illegible]	87
120	128	17 8	17 6	108	109	25	29	110	103
103	110	13,7	12,8	133	126	20	25	—	92
120	127	22,5	21 1	133	148	31	32	108	[illegible]
96,7	104,5	12,4	11 4	104	100	19	22	99[a]	91
—7,8		+1 0		+4		—3		+8	
<0,001		<0,01		> 0 05		<0,01		<0,05	
120,3	127,0	18,1	17,3	106	109	25[a]	28	110	104
—6,7		+0,8		—3		— 3		+6	
<0,001		<0 01		0,1		0.01		<0,01	

W m—THb A significant negati e correlation was found between an increase in W m after propranolol during th cath terization nd change in W between the work test before th catheterization nd the first test d ring the catheterization (P<0.01) The increase in W fter propranol l during the catheterization w highest n those who had a lower W t th f rst test du ng th catheterization as compared to before.

Discussion

The procedure of a cardiac cathet riz ti ma influence the results as t ma use variable degree of anxiety ertai p ient Bevegård *et al.* 1966 T oid this influence 10 to 15 min of rest f he thete is positioned in the pulmonary artery is recommended ntil ca diac p determined. The bjects of the present study were allowed to est for 30 min b f m determination was made Some

the subjects were a little tensed before and during the introduction of the cardiac catheter but this tension tended to disappear parallel to a decrease in pulse rate during the 30 min rest before the exercise started.

Holmgren, Jonsson and Sjöstrand (1960) found a small decrease insignificant, however in W_{170} between determinations before and during a catheterization in healthy subjects. A similar decrease in W_{170} was observed in the present study. The result indicates that 30 min of rest does not bring about basal conditions at a catheterization in all cases. The deviations were usually mall in the healthy subjects of the present study. It seems probable that the changes are of a regulatory origin. The correlation between the effect of propranolol on W_{170} and the difference between W_{170} before and at the 1 examination during the catheterization, supports this assumption. The effect of beta-blockade on W_{170} was also correlated to the resting pulse rate at the examination before the catheterization. A high rate at rest can be a sign of a regulatory disturbance for instance in patients with vasoregulatory asthenia (Holmgren *et al.* 1957).

After the first exercise in a sitting position the subjects rested for 60 min in a supine position until the second exercise was started. This interval was considered sufficient to enable the subjects to return to resting conditions after the exercise. An interval of 30—45 min has been used in some other investigations (Bevegård, Holmgren and Jonsson 1960 Bevegård 1963 Epstein *et al.* 1965) Epstein *et al.* (1965) reported no difference in the hemodynamics during maximal exercise (mean pulse rate 180 beat min in connection w th a cardiac catheterization before and after placebo in some healthy controls. It is reasonable to interpret the systematic differences rec rded in this tudy between the investigations during the catheterization as effect of prop anolol

In the present and previous studies (Furberg 1967a and b propranolol ha been used n oral dose of about 0.?? mg kg b wt. The increase in the heart rat that urs aft drene gic timulation with isoproterenol (1.5 μg min i.v. is blocked p 8) by propr nolol an oral dose of 0.?6 mg kg b wt. (Ablad *et al.* 1967)

The mean dec ase n pulse rat after propranolol during the first load was somewhat naller than during the second. This is in accordance with result reported by Chamberlain 196 H n ted that the blocking effect of propranolol t nded to increase w th rising he rt t d ring exercise. It i known that the ffect of anticholinergic dr gs p lse r t during w rk decreases progressively with ncreasing w rk loads R b n *et al.* 1 Bevegård 1963. The related report und ur results are n ac ordanc with th se from a tud by R binson *et al.* 1966) They pointed out that th se h art rat with mild exercise is mediated predominantly by decre se par mpathetic activit nd that ympathetic stimulation contributes to the d u eler tion at h gher levels of work. This is alid for young healthy subjects.

The absolute decrease in p se t d ring work bserved in the present tudy was smaller than that rep rt d lier Epstein *et al.* 1965 Cumming and Carr 1966 Robinson *et al.* 1966 and Ch mberlain 1966. They however used doses of

propranolol 3—5 times higher than ours.[1] The blocking effects of the drug are correlated to the dose (Gebhardt *et al.* 1965 Ablad *et al* 1967)

The beta-blockade induced a decrease of about 8 % in cardiac output on the first and of about 4 % on the second work load. The latter reduction was smaller than that of the pulse rate as some subjects had a slight increase in stroke ol me after propranolol. The slight reduction in cardiac output induced by propranolol was compensated for by a better oxygen utilization in the working muscles d ring the blockade. This is expressed as a larger a oxygen difference. An increased a- oxygen difference has been found after propranolol in previous hemodynamic studies. The increase of about 5 in the present study is about half of that calculated during maximal exercise in treadmill studies by Epstein *et al.* (1965) Results from exercise tests in sitting and supine positions are not comparable as there is a larger a-v oxygen difference in the former position (Bevegård, Holmgren and Jonsson 1960) A slight insignificant increase in the mean stroke volume as in th present study has been reported by most in estigators.

No change in oxygen ptake during work after propranolol was noted in our study It has been observed tha propranolol in higher doses slightly reduces the oxygen uptake during maximal exercise while it is unchanged during submaximal exercise (Epstein *et al* 1965) They also pointed to a decrease of about 40 % in exercise endurance on a work load that produced total exhaustion after 3 to 6 min during beta-blockade. Furberg (1967b) has found that the bility to perform hea y exercise was slightly decreased in athletes while it was somewhat increased after propranolol in subjects with signs of a hyperkinetic circulation. Similar results have been reported by Bollinger *et al.* (1965) These results indicate that the effect of propranolol on the exercise performance is influenced by the kinetics of the circu lation. Epstein *et al.* (1965) think that the fall in cardiac output after propranolol is incompletely compensated fo by an increase in the a oxygen difference at maximal levels of work, while it is fully compensated for at submaximal levels. It is probable that the effect of propranolol on the exercise performance is also question of dosage.

The effects of propranolol on the mean pressures in the pulmonary and brachial arteries are similar to those observed by Epstein *t al* (1965) nd Cumming and Carr (1966)

The increase in W after propra olol in the present study was about 15 Such an increase was to be expected (F rberg 1967b) judging from the data obtained before the catheterization. F rberg f und that subject w th a W within ±1 S.D from the regression lines, W —HV nd W —THb had a mean ncrease in W of about 20 % during beta-blockad while subject w th a W more than +1 S D from the W value calculated f om HV nd THb had a corresponding increase of about 10 %. The subjec of th prese t study belonged to these groups except for one case with a somewhat low W in relation t THb Fig 1) It is

[1] Equipotent oral and i. doses of propranolol thow be he io 3 1 (Johnsson, Norrby and Solvell 1967)

reasonable to believe that the changes in W_{17} after propranolol previously reported to occur in patients with a normo- or hypokinetic circulation are mainly due to changes in the a-v oxygen difference. The other main determinant of W_{17}, which is the stroke volume (Holmgren, Jonsson and Sjöstrand 1960), seems to be only insignificantly influenced by the beta-blockade.

A mean increase in W_{170} of about 80 % during beta blockade has been observed in patients with signs of vasoregulatory asthenia (VA) (Furberg 1965, 1967b). Bollinger, Gander and Forster (1965) have reported similar results in a step-test study of patients with high pulse rate at rest and low W_{170} in relation to THb. Arvedson, Furberg and Linderholm (1962, 1967) observed that ganglionic blockade caused a normalization of the low a-v oxygen difference and the high cardiac output in relation to the oxygen uptake while the normal stroke volume was uneffected in VA patients at rest and during exercise. These hemodynamic effects of the ganglionic blockade were similar although more pronounced than those occurring in healthy subjects after propranolol in the present study. Preliminary studies have shown that beta blockade and ganglionic blockade induce similar hemodynamic changes in VA patients (Furberg 1965, 1967a). A normalization of the hyperkinetic circulation during exercise also occurs after a period of physical training (Holmgren *et al.* 1959). In the related studies the exercise was performed in a supine position. No difference exists between W_{17} estimated in supine and sitting positions in VA patients (Holmgren *et al.* 1957). It seems probable that the effect of propranolol on W_{170} in a sitting position in patients with signs of a hyperkinetic circulation is mainly due to changes in the a-v oxygen difference. This means that the differences in effect of the beta-blockade on W_{170} between subjects with hyper-, normo- and hypokinetic circulation is probably only quantitative.

In a stud f patients with various psychiatric diseases closer relationships between W_{170} and th circulat ry dimensions, HV and THb have been shown after propranolol (Furberg 1967). In patients with chronic anxiety states and in psychotic tient a normalizat on of a hyperkinetic circulation at rest occurs during a beta-blockad (H rris 1965 nd 1966). It seems probable that the related changes in W_{17} in psy hiatric patie t fter propranolol may in principle be expla ed by the result f the present hem dy amic tudy. Thus it is l kely that th increase in W_{170} in most patient is mainl d t a larger a oxygen difference and only sl ghtly to an incr ase n troke ol me.

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Distribution and Metabolism of H³-atropine in Mice

By

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Abstract

Albanus, L., L. Hammarström, A. Sundwall, S. Ullberg and B. Vangbo *Distribution and metabolism of H -at pi n mice* Acta physiol. scand. 1968. *73* 447—456.

The distribution of tritium labelled atropine in the mouse has been studied with whole body autoradiography The proportions of unchanged atropine in different tissues were established with paper chromatography It was found that H³-atropine is rapidly taken up by the iris and ciliary body the lung, salivary glands and certain endocrine glands such as the pituitary thyroid, parathyroid, pancreatic islets and adrenal medulla. I is also rapidly accumulated in the secretory epithelium of the stomach and secreted into the lumen. Except for the choroid plexus and epiphysis ery low concentrations of radioactivity are present in the brain. Very little passed the placenta barrier into the foetus. Although atropine is extensively metabolized in the mouse almost all the radioactivity present in submandibular gland and stomach was in the form of unchanged atropine.

Although the metabolic disposition of atropine in animals has been studied by a number of investigators (see e.g. Ipsen 1906. Veit and Vogt 1935. Tönnesen 1948, Gosselin *et al.* 1955 Evertsbusch and Geiling 1956. Gabourel and Gosselin 1958, Kalser *et al.* 1957 Gosselin and Gabourel 1958 Werner and Schmidt 1959 Werner 1961, Albanus *et al.* 1968) little is known about its distribution in the body However some studies using C -labelled atropine and impulse counting have been performed. Gosselin *et al.* (1955) and Kalser *et al.* (1957) have found high concentrations of radioactivity in kidney liver bile small intestine and also in lung, testes and pancreas, but a low concentration the brain In addition, an autoradiographic study of selected organs has been performed by Werner *et al.* (1966)

In the present paper the distribution of tritium labelled atropine has been studied in the mouse by whole body autoradiography in order to further elucidate the distribution.

Since atropine is extensively metabolized in the mouse was also necessary to establish the identity of the radioactivity th tissues

Methods

[illegible]

Tritiated tropine (generally labelled) [illegible] specific activity of 491 mc/mmole [illegible] used. The tropine base was dissolved [illegible] dilute HCl (10 mc in 0.8 ml 0.05 N HCl)

Adult mice were used as experimental animals. Each animal was injected in a tail vein with 0.1 ml (500 μc) of the H [illegible] tropine solution. The dose corresponded to 20 mg/kg expressed as atropine sulphate. [illegible] one series of five female non-pregnant mice weighing about 20 g the animals were killed at the following times after injection 5 min, 20 min, 1 hr 4 hrs and [illegible] 4 hrs. Two pregnant mice in late gestation state (weight [illegible] g) were similarly injected ([illegible] 10 mg/kg) and killed after 30 min and 4 hrs.

The mice were anaesthetized with ether and killed by immersion in [illegible] mixture of hexane and solid carbon dioxide at about − [illegible] C. Sagittal 20 μ sections through the whole frozen animals were cut and dried at −15 C.

Autoradiographic exposure [illegible] was made by apposition against Ilford G5 nuclear plates (emulsion thickness 10μ) and Gevaert Sino X-ray film. The exposure time was 60 days. The autoradiographic method has been described in detail elsewhere (Ullberg 1954 1958)

[illegible]

Blood samples were taken by puncture of the infraorbital plexus with [illegible] micropipette. Urine was collected on [illegible] piece of filter paper after gentle pressure over the lower abdomen in order to initiate voiding. The radioactivity [illegible] in the paper strip was eluted with water.

Following decapitation the different tissues (organs) were dissected out, frozen in liquid nitrogen and ground to fine powder in a mortar.

1 g of ground tissue (or 1 ml of blood) was extracted with 2 ml acid ethanol (0.2 % acetic acid in 96 % ethanol) at room temperature for 30 min. After centrifugation the pellet was resuspended in 1 ml acid ethanol (0.15 % acetic acid in 70 % ethanol), extracted for 30 min and centrifuged. The second extraction was then repeated once. The combined supernatants [illegible] concentrated by evaporation [illegible] to a final volume of 0.2 ml and [illegible] centrifuged in order to remove the precipitate.

The tissue extracts were chromatographed together with unlabelled tropine on Whatman no. 1 filter paper in n-butanol [illegible] water acetic acid (5 5 1) upper phase. The radioactive metabolites were localized with Packard strip scanner and the unlabelled atropine with modified Dragendorff reagent.

Results

[illegible]

Following an intravenous injection of H^3 atropine the radioactivity was rapidly taken up by a number of tissues such as the submaxillary gland, Harderian gland, gastric mucosa, liver, lung and kidney (Fig. 1). It was also taken up in the ciliary body, iris and some endocrine organs, namely the pituitary, pineal body, thyroid, parathyroid, adrenal medulla and pancreatic islets. 5 min after injection the concentration in these tissues considerably exceeded that in blood and skeletal muscle. The specific accumulation in most of these organs was still visible after 1 hr but subsequently disappeared. The concentration in the brain and the spinal cord was low except for the choroid plexus and the [illegible]. The uptake in the foetus was [illegible] very low (Fig. 5).

The distribution in some of the tissues had a pattern which deserves a more detailed description.

[illegible] The [illegible] in the central nervous system was very low but some radioactivity was [illegible] rather evenly distributed (Fig. [illegible]). [illegible]

Purchased from the Radiochemical Centre, Amersham, England.

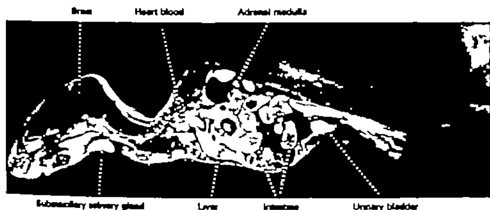


Fig. 1 Distribution of H³-atropine in mouse 5 min after injection. Note accumulation (light areas) in the salivary gland, pituitary and adrenal medulla.



Fig. 2 Detail of an autoradiogram showing the distribution in the head and neck region of mouse 5 min after injection of H³-atropine. Note the accumulation (light areas) in the salivary gland and the pituitary. Note also the uptake in the semilunar ganglion

higher concentration than the era of h t in w sec in the choroid plexa and also in the areas adjacent to the n les I th pineal body there was ptake which markedly exceeded that of the ula ig blood Also the conce tration in the spinal and sympathetic ganglia w h her han in th blood The oncent tion was higher in the areas with nerv ells d ar h nerv trunks

Ey — In the ey a rapid ptake w ed the iris and the ciliary bod



Fig 3 Detail of whole body autoradiogram showing the distribution of H^3-atropine in the ey and sur ounding tissues 20 min after i. i jection. Note the ptake (light areas) in the cillary body iris and Harderian gland.

3) There was the same high concentration in both the inner parts (sphincter muscle) and the peripheral part (dilatator muscle) In the ciliary body the radioacti ity was found n the whole organ. Some radioactivity was found in the aqueous humor In the eye region a strong accumulation was also seen in th Harderian gland.

Endocri e gans — Several of the endocrine glands rapidly accumulated H In the pituitary the radioactivity seemed to be confined to the neurohypophysis and the adenohypophysis whereas the concentration in the intermedial part was low (Fig 2)

In the thyroid a h gh concentration was observed in some spots scattered all over th gland possibly representing the so-called parafollicular cells.

The adrenal med lla showed a high concentration, which seemed to be most marked in some pots mainly localized in the peripheral parts of the edulla. The pancreatic islets had a high co ncentration of radioactiv substance (Fig 4)

Digestiv sy t m — The submandibular and parotid salivary glands showed about the highest c ncentr tion of H -atr op ne in the body Also the sublingual gland showed uptake of radioactiv t but the concentration was lower than in the ther salivary glands

The liver showed a pid ac ul tion and a nsible activ ty was noticeable also after 4 hrs. As arly as 5 min fter jection a ery h gh concentration was noted in the biliary ducts (Fig 1

In the stomach a high co ncent t was seen i the glandular mucosa but low in the oesophageal part which, in small rodents, is covered with a squamous non-secre-



Fig. 4 Detail of whole body autoradiogram showing the ptake (light reas) of H³-atropine in the pancreatic islets and adrenal medulla 20 min after L injection.

tory epithelium (Fig. 5) A secretion from the glandular portion into the gastric contents could be observed as early as 5 min after injection.

W thin the mucosa the highest concentration was seen n the basic and middle layer where the zymogenic and parietal cells are found h le the superficial layer covered by surface epithelial cells showed low concentration

The concentrations in the muscula l yer f the tomach and the mucosa and in the muscular layer of the intestine w slightl h gher than the concentration in the blood. In the intestinal lumen a considerable c ncentration was noted after survival periods longer than 30 min.

Respirat ry system — High conce tration the respir tory system was more pparent in the l ng portion consisting of l ng al e l and bronchioli while no marked accumulation in the mucosal lining or m scles of th large bronchioli or trachea was observed.

Radioactive metaboli n the ur l l ll t gen — After 2 hrs bout 50 per cent of the injected radioacti t w is rec red in the urine Pape hroma tography of the urine in n-butanol. cetic d water (5 3 1) rev led 5 ad o active pots (Fig 6) By addition of tri m labelled atropine t the urine t as established that one of the major peak Rf 0 77) was identical with ncha

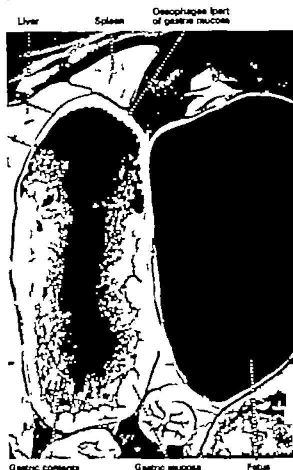


Fig. 5 Detail of whole body autoradiogram of pregnant mouse showing the distribution of H-atropine 30 min after injection. Not the peaks (light areas) in the basal layer of the gastric mucosa, the li and the pancreatic islets. Very little radioactivity in the fetus

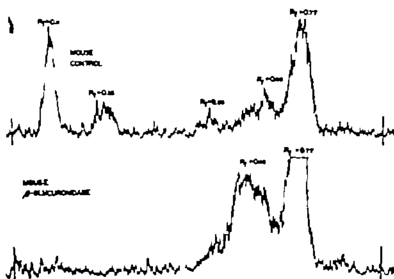


Fig. 6. Radioactiv metabolites in mouse ine following int avenou injection of H atropine Paper hromatography in n-butanol, water ac ic acid (5 5 1) upper phase

TABLE I Proportions of the different metabolities in mouse urine 2 hrs after intra enous injection of H³-atropine.

Rf	Before	After
	incubation with β-glucuronidase	
0.11	33 %	—
0.25	16 %	—
0.54	—	7 %
0.66	16	42
0.77	36	51 %

Urine incubated 20 hrs t 37° C with 100 units β-glucuronidase in aceta buffer pH 5.

atropine. After incubation of the urine w th β-glucuronidase, the two peaks with the Rf-values 0.11 and 0.25 disappeared while those with the Rf-values 0.66 and 0.77 increased (Fig 6) Radio assay of the eluted spots gave an estimate of proportions of the different metabolites in the urine (Table I)

When extracts from plasma, brain, salivary glands, lung stomach ileum and liver were chromatographed, marked differences in the distribution of radioactive metabolites became pparent. The results from a series of experiments are shown in Fig. 7 The animals were killed 20 min after injection. In all experiments unlabelled atropine was added to one half of extract before chromatography in order to detect ariations in Rf values and possible multiple spots. The salts present in the concentrated tissue extracts sometimes influenced the Rf values and caused tailing As seen in Fig. 7 the radioactivity present in l ng salivary gland, stomach and brain is mainly in the form of unchanged atropine while the liver contains mainly glucuronides. The metabolite pattern in the small intestine is more like that in th urine. Blood seems to contain about equal proportions of glucuronides nd unhanged atropine.

Discussion

According to the chromatographic separation of radioacti components in tissue extracts most of the radioactivit n salivary gland, h g brain and stomach repre sents unchanged atropine. The major part of th rinary radioactiv ty was in the form of glucuronides and nchanged tropine which is in greement w th earlier studies (Gabourel and Gosel 1957)

In thorough tudy of the disposition of radio. tiv ty in mice follow g injection of C labelled tropine E ertsbusch and Gerling 1956) found that 85 per cent was excreted in the urine 13 per cent w th the f eces and 1.5 per cent th expired air \ biliary excretion is indicated in th prese t experiments since th bile canaliculi were radioactive within 5 min after jection As the major part of the radioactivity

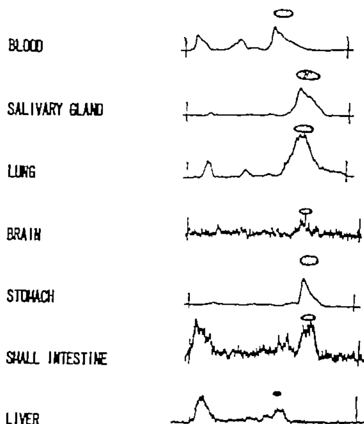


Fig. 7 Radioacti metabolites in different tissues follo ing intravenous injection of ^{3}H-atropine Carrie atropine was added t the tissue extract before paper hromatogr phy in n-butanol, w ter acetic acid (5 5 1) pper phase The carrier was sualized with modified dorff reagen

in the li er is in th form of metabolites, probably only small amounts of nchanged atropine re excr ted in th bile According to Evertsbusch and Geiling (1956) a substantial amount of the radioacti ty excreted in the bile is reabsorbed in the intestine Thus al indicated by the a toradiographic studies by W rner t al. (1966 who fou nd high rad act ity in the lymph essels of the intest ial illi. According to the hromat grams, the small intestine (plus contents) contain metabolites nd nchanged tropine in about equal proportions.

Accumulation of t opine n th ga tric wall fter intravenous jection was noticed by Tonnesen rly a 1948, nd high concentration of rad activity in the cont nts of the st mach w f nd by Gossel n t al (19) follow g jection of C labelled atropine Th fact that i the a toradiogram there is an accumulation in the secreting epitheli m d n t in the non-sec ting esophage l part strongly suggests that the accum lat n mediated through an acti process, but non ionic diffusion probably contributes t the secretion nto the gastric ontent Th view is supported by the fact that Ton esen (1948) demonstr ted that the ystemic

absorption of atropine from the stomach is pH-dependent. The renal elimination of atropine has also been shown to be influenced by the pH of the urine (Albanus *et al.* 1968).

When interpreting the autoradiograms it is of interest to discuss the distribution of labelled atropine in relation to the known pharmacological effects. In the eye a strong accumulation of radioactivity is present in the ciliary body and the iris, which are well-known cholinergic structures inhibited by atropine. However the distribution within the iris did not seem to be confined only to the sphincter muscle but also comprised to the dilator part. In this connection it may be mentioned that catecholamines are also present, not only in the dilator muscles but also in th circular muscles (Malmfors 1965). The other organ in the eye region where a strong accumulation of H atropine is found, the Harderian gland, is also cholinergically innervated and the secretion of red tears is blocked by atropine.

The accumulation in the serous salivary glands also agrees with the efficiency of atropine to block salivary secretion. E en certain endocrine glands such as the pituitary thyroid, parathyroid, pancreatic islets and drenal medulla, accumulate relatively large amounts of atropine and literature data reveal pharmacodynamic effects of atropine on these glands (Guillemin 1955 Shvedov 1961 Mordi 1963 Robinson 1955). In this connection it is interesting to not that exogenous serotonin and catecholamines are also taken up by these organs and it has been proposed that biogenic amines may play a role in the regulation of synthesis and or release of hormones (Ritzén *et al.* 1965 Pearse 1966, Ullberg and Hammarström 1967).

On the other hand, rather low concentrations were found in heart and smooth muscles although their function is affected by small doses of atropine.

The low concentration i the brain, however is compatible with the fact that the pharmacological effects on the central nervous ystem are seen only after high doses.

The results of the present paper indicate that atropine is specifically taken up in a number of tissues where t is known to exert a pharmacologic effect and where cholinergic receptors are assumed to occur. It must be remembered, however that apparent discrepancies ha e also been encountered (*g* smooth muscle nd lung) where the autoradiographic pict re does not correlat well w th cholinergic f nction.

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Amine Formation by Rat Mast Cells *in vitro*

By

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Abstract

SLORACH, S. A. and B. UVNÄS, *Amin f rmation by at mast cells in it o*. Acta physiol. scand. 1968. 73 457—470.

The effects of incubation temperature, time, pH, cell concentration, pyridoxal and aminoguanidine on ^{14}C histamine formation by rat mast cells   *itro* were investigated. I was shown that disruption of the cells by physical means (sonication, freezing and thawing and hypotonic shock) or treatment with the histamine releaser ATP caused marked reduction in histamine formation. I contrast to this, treatment of the cells with compound 48/80 caused an increase in histamine formation. After w ter lysis and centrifugation the histidine decarboxylase activity was found predominantly in the non-particulate fraction. The cells were also show to decarboxylate 5HTP and dopa *vit* forming 5HT and dopamine. H droxylation of phenylalanine, tyrosine or tryptophan could not be demonstrated. This suggests that the reason by normal rat peritoneal and pleural mast cells contain relatively low amounts of 5HT (compared to histamine) and no dopamine is the unavailability of the immediate precursors (5HTP and dopa) and their inability to synthesize these by hydroxylation of tryptophan or tyrosine.

Normal rat peritoneal mast cells contain large quantities (~30 μg per million cells) of histamine and smaller quantities (~1 μg per million cells) of 5-hydroxytrypt amine (5HT) (Moran, U nås and Westerholm 1962). To our knowledge dopamine has not been detected in these cells.

Isolated rat peritoneal mast cells ha e th capacity to take p and decarboxylat histidine (Schayer 1956, Rothschild and Schayer 1959) and 5-hydroxytryptophan (5HTP) (Lagunoff and Benditt 1959) forming histamine and 5HT respecti ely. Although dopa decarboxylase has been demonstrated in neoplastic murine mast cells (Hagen t al. 1960) it has not been demonstrated in normal rat mast cells.

Furano and Green (1964) showed that mast cells from the peritoneal fluid of normal rats were able to take p and retain histamine and 5HT both *vivo* nd *vitr*, but were unable to take up dopamine or noradrenaline. Thus the histamine and 5HT present in these mast cells can either be formed there or formed else wher in the animal (or derived from ts food) and merely taken up and tored by the mast cells. The ability of the cells to synthesize the amines themsel es will of course be greatly influenced by the ailability of the appropriate substrate.

In the work presented here we have tried to determine why normal rat mast cells, which contain large amounts of histamine, contain such relatively small quantities of 5HT and no dopamine. We have compared the capacities of the mast cells to decarboxylate histidine, 5HTP and dopa *in vitro*. In addition, the ability of such cells to hydroxylate precursors of the latter two substrates i.e. tryptophan, phenylalanine and tyrosine has been examined. Earlier work by Day and Green (196) using neoplastic murine mast cells showed that these cells could hydroxylate tryptophan but not phenylalanine or tyrosine. However Levine, Lovenberg and Sjoerdsma (1964) using cells derived from the same strain of cells, in addition to confirming the tryptophan hydroxylating ability also demonstrated the presence of a phenylalanine hydroxylase. It has not however been shown, even in these neoplastic cells, that dopa, the immediate precursor of dopamine, can be formed from phenylalanine or tyrosine.

It has been demonstrated that in rat peritoneal mast cells histamine is stored in discrete intracellular granules together with protein and heparin (Thon and Uvnäs 1966). Compound 48/80 causes degranulation, an energy requiring process, after which the histamine can be released from the extruded granules by a simple ion-exchange with cations in the surrounding medium (Thon and Uvnäs 1967). In view of the earlier hypothesis by Schayer, Rothschild and Bizony (1959) that disruption of mast cells with compound 48/80 would probably cause loss of the histidine decarboxylase located (presumably) in the cell sap, we investigated the effect of compound 48/80 on the histamine forming capacity of mast cells *in vitro*. This effect was compared with that of another histamine liberator ATP. From these experiments and experiments in which the mast cells were disrupted by physical means and then fractionated by centrifugation it was hoped to obtain information about the site of histamine formation in rat mast cells.

Methods and Materials

[illegible]

[illegible] cells were obtained from the peritoneal and pleural cavities of male Sprague-Dawley rats ([illegible] 400 g) and separated by density gradient centrifugation in Ficoll according to the method of Thon and Uvnäs (1966). The cells were not exposed [illegible] sucrose [illegible]. Cell counts were performed [illegible] Bürker chamber after staining with toluidine blue.

Incubation procedure [illegible] general method

Mast cells were incubated [illegible] buffered salt solution (NaCl 154 mM, KCl [illegible] 7 mM, CaCl 0.9 mM) containing 10 [illegible] Sorensen phosphate buffer (Na2HPO + KH PO [illegible] 6.7 × 10 [illegible] M, pH 7.[illegible]) and 1 mg bovine serum albumin per ml. The incubation mixtures also contained pyridoxal phosphate 10 [illegible] M and the substrate (concentrations given in the Results). Unless otherwise indicated the incubations were carried out in a final volume of 2 ml [illegible] 37 °C for 3 hrs in glass-stoppered tubes. The tubes were shaken intermittently during the incubation period. In each experiment "blank" incubations were run using mast cell suspensions heated at 80 °C for 10 min before the addition of the substrate. Substrates were [illegible] added last to the incubation mixtures, immediately prior [illegible] commencing the incubation. When used, other reagents (aminoguanidine, ATP, NSD 1 [illegible], [illegible]-methyl dopa and compound 48/80) were dissolved in the buffered salt solution adjusted [illegible] pH 7.2 when necessary and added to the mast cell suspension immediately before adding the [illegible].

[illegible]

Ion-exchange Chromatography

After incubation, the tubes were heated [illegible] water bath [illegible] 80 °C for 10 min and 3 ml of

distilled ater as then added to each tube. 4 ml of the diluted incubate as added to 6 ml column (column diameter 1 cm) of Dowex—1X8, acetate-hydroxide form prepared accordin to Kremzner and Wilson (1961) (Originally 50—100 mesh resin was used but this later replaced ith 100—200 mesh resin, giving slightly improved esults) The columns er eluted ith Sorensen phosphate buffer (0.06 M) pH 7.2 diluted w th an equal olume of ater. The first 1.5 ml of eluate was rejected and the next 10 ml was collected olumetric flask.

Liquid scintillation count g

The radioactivity in 0.5 ml samples of the eluates and the diluted incubates was determined after dissolution in 15 ml of scintillator fluid, using Tri-Carb liquid scintillation spectrometer Packard Instrument Co., Inc., La Grange Ill., USA. The scintillator fluid consisted of so- lution of 0.4 % 2,5-diphenyl-oxazole and 0.01 % 1 4-bis-2 (4-methyl-5-phenyloxazolyl) benzene in a mixture of equal olumes of toluene and ethylene glycol monoethyl ether

Calculation of histamine formation

The quantity of histamine formed was determined from the percentage of C histidine con- erted to histamine This was calculated from the formula Percentage conversion = (Radio- activity (cpm) per ml of eluate $\times 2.5 \times 100$)/(Radioactivity (cpm) per ml of diluted in- cubate)

From knowledge of the mast cell concentration, the quantity of histidine and the per centage conversion in the incubates, the histamine formation per million cells i 3 hrs was calculated.

Radio paper h omatography

Ascending paper chromatography was carried ou on Whatman no. 1 paper marked out in strips 4 cm ide. After drying, the chromatograms were cut into 1 cm lengths and, after cutting into smaller pieces, these ere placed in counting ials together ith 15 ml of scintil- lator solution and the radioactiv ty was measured as bove. Reference chromatograms were run either using non-isotopic referenc substances and then staining w th ninhydrin or using ^{14}C labelled reference compounds and proceeding as abov The following solvent systems were used.

n-butanol acetic acid water 12 3 5, (All ratios by olume) isopropanol ammonium hydroxide (d. 0.88) water 20 1 2 and n-butanol pyridine water 1 1 1 (Hereafter referred to as B.Ac.W I.A.W and B.P.W) The purity of all isotopic substrates used in these experiments as checked by paper chromatography

Re overy f histamine from th io -excha ge ol mns

Non-isotopic histamine was determined by fluorimetry after condensation with o-phthal- aldehyde, according to the method of Kremzner and Wilson (1961)

The recovery of added histamine, after separation from histidine using the ion-exchange column method, was 86—92 %. The recovery was determined using both isotopic and non- isotopic methods.

Confirmation that histamin was the aly ng fac t adioac n ompo d in the ion-excha ge olum eluat

After incubation (L-^{14}C-histidine 5×10 M pyridoxal 2×10 M mast cells 0.185×10^{6} per ml), and ion-exchange separation, 50 µl samples of the eluates ere chromatographed on paper Three different solvent systems, namely B.Ac.W I.A.W and B.P.W ere used Each chromatogram showed single peak corresponding to the histamine peak on the ref ence chromatograms.

Cell disruption proced

( ) Distilled water The mast cell suspension was cent f ged 350 g for 10 min and the supernatant was poured off and remov d as ompletely as possible by the use of small strips of filter paper Distilled water was then added to olume equal half ha of the original suspension, the cells ere resuspended nd the suspension was shaken frequently during 10 min. An equal olume of double st ngth buffered sal olution was then added to the sus- pension.

b) Sonication Disruption of the st ell rried ou plastic centrif ge surrounded by ice-water using an M.S.E. l asonic disintegrator model 60 W

) Freezing and thawing This ied ou l rapidl freezing the ll su ethanol-solid arbon dioxide and allow ie ha lowly

Isolatio f ^{14}C dopaml by ion-e hange h omatogr phy

After incubation, heating at 90 C of 5 min and removing the samples for paper hromatography 4 4 ml of 0.33 % perchloric acid was added to each incubate After centrifugation, to remov the precipitated protein, and the addition of ascorbic acid (5 mg) the supernatant was adjusted to pH 6.5 with potassium carbonate solution (2N) Two ml of the resulting solution was added to a 500 mg column of Dowex 50W×4 Na form, previously treated ith M sodium acetate-acetic acid buffer pH 6.5 (10 ml) followed by distilled wate (5 ml) The olumn was eluted with Sörensen phosphate buffer (0 06 M) pH 6.5 (30 ml) followed by 0 1 % EDTA solution pH 6.5 (10 ml) These eluates were rejected. The column as then eluted with 2N hydrochloric acid (10 ml) and the radioactiv ty in 0.5 ml aliquots of this eluat (ontaining the dopamine fraction) was determined by liquid scintillation pectrometry aft dissolution in 15 ml of scintillator fluid. The radioactivity in the solution applied to the olumn was also determined and the quantity of dopamine formed per million mast ells was lculated in manner analogous to that used for the histamine experiments (*id supr*)

The ion-exchange procedure used f this experiment is similar to that published by Häkanson and Moller (1963)

Comparise f ^{14}C ty ou e d C dopa metabolism i ai mast lls i I bations

Mast cells (366,000 per ml) were ncubated with DL- ^{14}C-tyrosine (2×10 M) or DL- ^{14}C-dopa (2×10 M) in the presenc of pyridoxal (10 M) The incubations w e carried out protected from light in 1 ml of buffered salt solution (pH 7.2) containing 1 mg serum albumin per ml, for 4 hrs t 37 C After incubation, 4 ml of 0.33 % perchloric acid was added to ach tube and the resulti g precipitates were removed by centrifugation.

(a Ty sin bat

4 ml samples of the supernatants were dil ted w th 20 ml of 0.1 % EDTA sol tion pH 6.6 and 10 mg of sodium metabisulphite and 400 mg of alumina were added to each sol tion. The suspension was stirred mechanically adjusted to pH 8.6 with sodium hydroxide solution and then tirred for a further 10 min at this pH The suspension was added to olumn (20 cm × 1 cm d containing 400 mg of alumina and the olumn was then eluted w th 0.. M sodium acetat (10 ml 0 1 % EDTA solution pH 6.6 (10 ml) and finally with distilled water (10 ml) These eluates, which did not contain any techols, ere rejected. The columns were hen eluted with 0 2 N HCl (10 ml) ollecting the luates i 50 ml beakers containing 0 1 ml of 1 % ascorbic acid solution The acid luates, containing he echols, w re trea ed as described unde the separ tion of the dopa inculates (d f)

(b) Dopa i bat

ml samples of he per hloric acid supernatants prepared from the dopa incubations ere ded t 50 ml beakers ontaining 0 1 ml of 1 % ascorbic acid solution These solutions ere diluted w th distilled w ter 6 ml) and then treated in the same manner as he acid eluates from he alumina olumns The on ents of ach beaker were adjusted to pH w th sodium h droxide and then added to 6 m long columns (d 1 cm) of Dowex 50W×4 N form, 200 –400 mesh previously ea ed w th M sodium acetate acetic acid buff pH 6 6 (15 ml) followed by distilled wat 5 ml The olumns w re el ted with 0.1 % EDTA solution pH 6 6 (10 ml) Sorensen phospha buffer (0 06 M) pH 6 5 (10 ml) distilled ater (10 ml) and N HCl (4 ml) These luates contained dopa and wer rejected. Each olumn as then eluted w th N HCl 1 ml and the eluate (ontaining the noradrenaline fra tion) was ollected in glass ial suitable for liquid sc tillation spectrometry F nall ach olumn as luted w th N HCl 17 ml his eluate containing the dopamine fraction) as collected in second glass ial The luates in he glass ials were evaporated to dryness an oven at 0 C. The residues were dissolved in 0 5 ml of distilled w ter and 15 ml of scintillator solution and the adioactiv ty was de ermined by liquid scintillation spectrometry *ide supr*) The radioactivity as also determined samples of the solutions pplied to he ion-exchange olumns, enabling alc lation of the per entage on ersion of dopa to dopamine and hence the quantity of dopamine formed F om he adioac measurements on the perchloric acid solutions derived from the ty osine incubates and the adioacti ty in the dopamine-containing eluates the percentage conversion of osine dopamine was alculated The abov methods for separating the metabolites of osine and dopa has been developed by Nyluck and Sedall (t be published) from the original methods of Anton and Sayre (1962 and Murac ber et al. (1966)

In order to elimina e inaccu acies arisin f om impuri ies the adioacti substrates and/or non-enzymatic hanges taking plac du ing he incubations or other procedures, heat treated blanks were al ay used in hese experiments Duplic samples wer ru in ach

experiment and in cases where the coefficient of ariation bet een the duplic tes xceeded 7.5 % the results ere rejected. Each experiment was performed at least twice, unles othe ise stated. More detailed methods for certain types of experiment and any ariations f om the general method are given in Results. All amine alues are expressed as the f ee base

Materials

Ficoll as obtained from AB Pharmacia, Uppsala, Sweden, and human serum albumin (f ee from preser tives) from AB Kabi, Strängnäs, Sweden.

Compound 48/80 was kindly supplied by Dr B. Hogberg, AB Leo Hälsingborg Sweden

2 5-diphenyloxazole and 1 4-bis- (4-methyl 5-phenyloxazolyl)) benzen were obtained from the Packard Instrument Co., Inc., La Grange, Ill. USA.

NSD 1055 (4-bromo-3-hydroxybenzylo yamine phosphat) was kindly supplied by Mr D J Drain, Smith and Nephew Research Ltd., Gilston Park, England.

The following substances wer obtained from the Radiochemical Centre Amersham, England.

L-histidine (ring- ^{14}C) Sp act. 41.5 mc/mM and 37.8 mc/mM, DL-5-hydroxytryptophan (methylene-2 ^{14}C) Sp.act. 21.8 mc/mM DL-3-(3,4 dihydroxyphenyl)alanine- ^{14}C. Sp.act 31 2 mc/mM, DL-tyrosine 2- ^{14}C Sp.act., 50 mc/mM L-tryptophan (methylene ^{14}C) Sp.act. 14 7 mc/mM DL-3-phenyl (alanine-2 ^{14}C) 14 7 mc/mM All other substances wer obtained from usual commercial sources.

Results

Factor affecting ^{14}C histamine format on by mast cells in vit o

The ^{14}C histidine concentration used was 6×10^{-6} M or 10 M and the concentra tion of mast cells was betw een 90,000 and 500,000 cells per ml of incubate. Histamine was determined after ion-exchange separation. The histamine formation by normal cells, using the general procedure for incubation, was usually in the range 20 ng—40 ng (exceptionally as high as 65 ng) per million cells in 3 hrs.

Temperature—histamine formation at 27 C was 40 % of that at 37 C t 47 C it was 81 % of the 37 C alue

pH—the pH of the incubation mixtures was aried by altering the composition of the Sörensen phosphate buffer Before the ion-exchange separation the incubates were adjusted to pH 7.2. At the substrate concentration st died no sharp pH optimum was found over the range examined (pH 6 5—7 9) Activity was optimal over the range pH 6 85—7.5

C ll ncentration—the effect of ary g the cell concentration is shown in F g 1 It should be pointed out that the incubation mi t res wer not shaken continuously

Incubation t me—the influenc of ti ne on histamine f rmation is shown in Fig 2

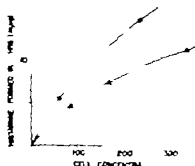


Fig. 1 Effect of cell concentration on ^{14}C hist amine formation by mast cells in vitro

Expt. I. ○—○—

Expt. II. △—△—

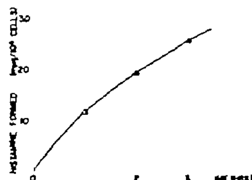


Fig. 2. Effect of incubation time on ¹⁴C histamine formation by mast cells in vitro.

Pyridoxal—the addition of pyridoxal phosphate to a final concentration of 10 M increased histamine formation by 13 No further increase was found when the concentration was increased to 10 M.

Aminoguanidine—in concentrations of 10 M and 10 M did not significantly affect histamine formation

Cell disruption—(a) Distilled water at 0 C, 20 C, or 37 C reduced the histamine formation to 14—27 % of that in untreated cells.

(b) Freezing and thawing reduced histamine formation to 6 of that in untreated cells

(c Sonication for 20 sec or 40 sec reduced the histamine formation t 21 and 18 respectively of that in untreated cell (single experiment)

am l aser

(a) *ATP* The expos re of mast cells t concentrations of ATP causing substantial ease of histamine from such cells caused a decrease in their histamine formation. Table I show the percentage histamine formation (compared with u tre ted cells) and the percentage histamine release after exposure to arious concentrations of ATP The release data re from Diamant and Kruger 1967)

(b *Compound 48/80* The exposure of mast cells to compound 48/80 in concentrations a sing histamine release did n t decrease their histamine forming capacity On the c ntrary ther w small but consistent increase in the amount of histamine formed Table I show the histamine formation (as a percentage of that in untreated lls in ma t ell t eated with rious concentrations of 48/80, together w th the histamine rele se t these c ncentrations. (Th latter data from Thon and U nas 1967

Decarboxylase i h b t The nhibit ry ffects of NSD 1055 and α-methyl dopa are shown in Table II

Ag (at) of at —Tabl III sh w the histamine contents of and the ¹⁴C histamine formation by mast ell taken f m male Sprague-Dawley rats f different ages. Unlike the gener l procedure l c lls obtained from the peritoneal and pleural

TABLE I. Histamine release from and histamine formation by rat mast cells treated with ompou 48/80 or ATP

ATP Conc.	48/80 Conc. (μg/ml)	Histamine release[a]	Histamine formation
	0.1	25	102 %
	0.2	47	103 %
	0.5	57 %	105 %
	1	63	113 %
	2	63	110 %
	5	63 %	112 %
	10	63 %	109 %
	20	63 %	116 %
10[illegible] M		0 %	97 %
10[illegible] M		70 %	47 %
10[illegible] M		70 %	37

[a] percentage of the total histamine content of the cells. 48/80 data from Thon and U as (1967) and Thon (pers. comm.) ATP data from Diamant and Krüger (1967)
[b] percentage of that in untreated cells.

TABLE II. Inhibitory effects of -methyl dopa and NSD 1055 on histamine formation by mast cells in vitro

Inhibitor	Concentration	Inhibition of histamine formation
α-methyl dopa	10[illegible] M	100 %
α-methyl dopa	10[illegible] M	77 %
α-methyl dopa	10[illegible] M	41 %
α-methyl dopa	10[illegible] M	16
NSD 1055	10[illegible] M	100
NSD 1055	10[illegible] M	91 %
NSD 1055	10[illegible] M	43
NSD 1055	10[illegible] M	3 %

Percentage reduction of histamine formation during hree hours. The results are the mea lues from duplicate experiments

tities were used in th ncubations, without sepa tion of the mast cell by the Ficoll gradient techniq There was no ppare t or lat on between the ge of th rats from which the ells were taken and the rate of hist m ne formation There as, however an increase in the mean hista nine t of the c lls, probably re flecting the increase their size

Localization of histidine d arbo ylase l ly thin th ma t ll

Mast cells were disrupted with distilled w t ror temperat e The di cells were then centrif ged t 2000 g f 0 min nd th e l ta

TABLE III Effect of ge and weight of rats on in vitro histamine formation by mast cells

Age (weeks)	5 1 2	6 1	9 1 2	10	11 1 2	12 1 2
Number of rats	4	4	4	3	3	2
Weight (mean, g	144	172	315	319	351	402.5
Cell conc (10⁶ in incubates	0.381	0.361	0.365	0.466	0.402	0.287
Histamine formation	0.044	0.0306	0.0269	0.034	0.0286	0.0307
Histamine content	18.4	18.3	37.8	36.9	33	55

µg per million cells in 3 hrs, 37 C, pH 7.2, histidine 10⁻ M, pyridoxal 10⁻⁴ M.
µg per million cells.

TABLE IV Relat histamine forming capacities of whole mast cells, w ter-lysed cells and fractions thereof.

Expt	Cell conc 10⁶ ml	Histamine formed ng 10⁶ whole ells	Whole cells	W ter-lysed cells: Before entr	2 000 g S N	2,000 g PPT
1	0 09	59	100	27	27	5
	0 1.6	44	100	20	49	5
					165,000 g S.N.	165,000 g PPT
3	0 123	0	100	4	17	0

L-Histidine oncen ion 10 M
xal Phospha ne 10 M
bation 3 hrs 3 C, pH

S.N = Supernatant
PPT Precipitate

capac ties of the precipitat th supernatant, th unfractionated mixture and normal mast cells wer ompared A similar experiment was performed using a higher centrifugal for 165,000 g f 30 min t fractionate the disrupted cells. The results of these experiment re gi en in Table IV The enzymatic ctivity remaining after water disr ption wa f nd predominantly in th supernatant Little or no activity was found in the pr 2000 g fraction.

C mpa iso of d ca bo ylat f C h tid n ¹⁴C dopa and ¹⁴C -hyd oxytryptophan by mast lls i t

Incubations were carried out u g L-¹⁴C-histidine DL-¹⁴C-5HTP and DL-¹⁴C-dopa as substrates. The inc bat ns wer perf rmed protected from light in a final v lume of 0.5 ml for 4 hrs at 37 C The incubations were terminated by heating at 90 C for 5 min and then 20 µl samples of the incubates were chromatographed on paper using the I AW sol ent stem The distribution of radioactivity on three

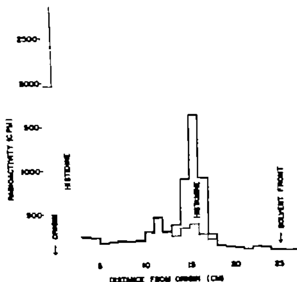


Fig. 3

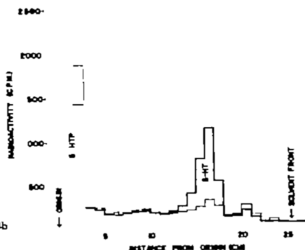


Fig. 3b

such chromatograms is shown in Fig 3a b and c From the percentage of the total radioactivity in each chromatogram th histamine dopamine or 5HT peak the quantities of these amines formed in the incubat ns were calculated (Values from the chromatograms of the "blank ncubates being deducted) Th quantities of histamine nd dopamine formed w lso determ ed by liq id scintillation count ing after separation using ion-exchange proced r d Methods) Th quan of amines formed and the substrat conc t tion used re presented in T b.

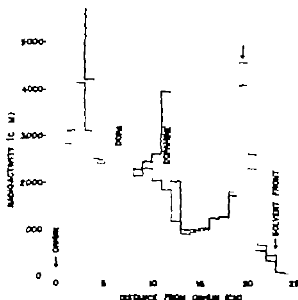


Fig. 3a, 3b, 3c. Radio paper chromatograms of incubation mixtures of mast cells and ¹⁴C-histidine, ¹⁴C-5-hydroxytryptophan and ¹⁴C-dopa respectively. The unidentified peak A (Fig. 3c) present in both incubates, contained 2.8 % of the total radioacti ty in each chromatogram. Full lines—incubates th normal lls. Broken lines—incubates ith heat-inacti ted cells.

TABLE V. A mparison of the decarboxylation of ¹⁴C-histidine, ¹⁴C-dopa and C 5-hydroxytryptophan by ra mast cells in vitro

Expt.	Separation method	Substrat	Cell conc. (10⁴/ml)	Product mμ moles /10⁶ cells per 4 hrs, 37° C pH 7.2	Mol. ratio of products.
	P per	L-histidine 2 10⁻⁴ M		8.4	1
		DL-5HTP 4 10⁻⁴ M		9.8	1 2
		DL-Dopa 4 10⁻⁴ M		18.7	2 2
	Ion exch.	L-histidine 2 10⁻ M	291	6.7	1
		DL-Dopa 4 10⁻⁴ M		12.1	1 8
2	P per	L-Histidine 2 10 M	613	4.1	1
		DL-5HTP 4 10 M		6.5	1 6
3	Ion exch	L-Histidine 2 10 M	563	2.56	1
		L-Dopa 4 10 M		4.59	1 8

Attempt to d m nst at C-t ypt phan metabolism in mast cells n t o

Mast cells (339 000 ml were inc bated w th L- ¹⁴C-trypt phan (10 M) in a manner analogous to that used n th abov experiment. After incubation the tubes were heated at 90 C for 5 min and 20 μl samples of the incubates were then chromatographed on paper using th I A W sol ent ystem. No formation of 5HT or tryptamine was detected

Attempt to dem nst at ¹⁴C ph nylala metabolism by mast cells in vit o

Mast cells (10⁶ ml) were incubated w th DL-¹⁴C 3-phenyl(alanine) (4×10 M

under conditions similar to those used in the previous experiment. After incubation the tubes were heated at 90° C for 5 min and then 20μl samples of the incubate were chromatographed on paper using the B.Ac.W. solvent system. No tyramine or phenylethylamine formation was detected.

Attempt to demonstrate ¹⁴C-tyrosine metabolism by mast cells in vitro. Comparison with ¹⁴C-dopa metabolism

Mast cells (572,000 ml) were incubated with DL-^{14}C-tyrosine (2×10^{-3} M) and the incubates were separated by paper chromatography in a manner similar to that described above for ^{14}C-tryptophan. No dopa, dopamine or tyramine was detected.

A further experiment was performed to compare the formation of dopamine in incubates of mast cells with C-tyrosine and ^{14}C-dopa. (Details are given under Methods.) It was found that no dopamine formation took place when the substrate was tyrosine. When the substrate was dopa 17 7 ng of dopamine was formed per million cells under the specified conditions in 4 hrs. Dopamine was not further metabolised to noradrenaline.

Discussion

The ion-exchange separation of C histamine from C histidine, followed by liquid scintillation counting provides a convenient and comparatively rapid method for measuring the histamine formed by mast cells in vitro. The paper chromatograms of the eluates showed that histamine was the only significant product eluted from the columns. The recovery of histamine from the columns was high and consistent.

Exposure of mast cells to sucrose was deliberately avoided following the reports by both Rothschild and Schayer (1959) and Lagunoff and Benditt (1959) that the use of differential centrifugation in hypertonic sucrose, as a method of separating mast cells from other peritoneal cells, gave preparations having little or no histidine decarboxylase or 5-hydroxytryptophan decarboxylase activity.

The influences of pH, temperature, incubation time and pyridoxal on histamine formation by mast cells are broadly similar to those reported by Schayer (1956) and Rothschild and Schayer (1959).

The histaminase inhibitor aminoguanidine did not have any effect on the amount of histamine produced during the routine incubation period. This strongly suggests that rat mast cells do not contain histaminase. Day and Green (1962) reported that neoplastic murine mast cells did not catabolise histamine and we have not found any reports of the presence of histaminase in rat peritoneal mast cells.

Rothschild and Schayer (1959) showed the presence of histidine decarboxylase in the 25,000 × g supernatant after disrupting rat mast cells by freezing and thawing. Hagen *et al.* (1960) reported that the histidine decarboxylating activity of homogenates of mouse mast cell tumours separated by differential centrifugation was localized in the non-particulate cytoplasmic material. Our results show that after disruption of the cells with distilled water and centrifugal separation the enzyme activity appears predominantly in the non-particulate fraction. Because of

solubility of the enzyme it is difficult to know whether in the intact cell it is present in the cytoplasm or if it is structure-bound and only found in the non-particulate fraction after disruption due to dissolution during the disruption and separation procedures.

Schayer Rothschild and Bizony (1959) believed that the high histidine decarboxylase activity found in the skin of rats injected with compound 48/80 was probably associated with the new resistant mast cells produced as a result of this treatment. They also assumed that disruption of the mast cell with compound 48/80 would release histidine decarboxylase which they believed to be in the cell sap. If this were so we would have expected a pronounced decrease in histamine formation by mast cells treated with 48/80 *in vitro* whereas in our experiments the reverse occurs. Our findings are that exposure to compound 48/80 also causes an increase in histamine formation in normal mast cells.

Disruption of mast cells by sonication freezing and thawing (previously reported by Schayer 1956) or lysis with distilled water causes a large reduction in the histamine forming capacity of the cells. Thus it would seem reasonable to conclude that degranulation of the cells with compound 48/80 does not affect the cell wall in such a way that the enzyme is released from the cell nor does it produce any gross changes in the fundamental integrity of the histamine producing mechanism. Furthermore since a large proportion of the intracellular granules is extruded from the mast cells on exposure to compound 48/80 it is unlikely that the histidine decarboxylase is located in the granules. This is supported by the results of the experiments where the cell fractions were separated by differential centrifugation after lysis.

It has been shown (Diamant and Krüger 1967) that ATP releases histamine from isolated mast cells without concomitant degranulation. The present finding that the histamine releasing action of ATP is accompanied by a marked decrease in the histamine forming capacity of the cell supports the suggestion that histamine release by ATP is accompanied by gross changes in the cell.

The inhibition of histamine formation caused by NSD 1055 is due to inhibition at the decarboxylation stage since Cabut and Haegermark (1968) have shown that concentrations of this compound causing almost complete inhibition of histamine formation did not prevent the uptake of substantial quantities of histidine.

Levine, Sato and Sjoerdsma (1965) reported that the administration of NSD 1055 *in vivo* to rats in concentrations which markedly reduced the histamine levels in heart stomach and urine did not alter the histamine levels in the peritoneal mast cells. However the cells were taken only 3 hrs or 4 hrs after administration of the inhibitor and since the turnover of histamine in peritoneal mast cells is probably extremely slow the level of histamine in the cells will change only very slowly although the synthesis may be almost completely inhibited.

Levine (1966) demonstrated that the administration of NSD 1055 to humans having systemic mastocytosis markedly reduced the symptoms attributed to histamine and prevented symptomatic exacerbation associated with histidine loading. Thus it

appears that NSD 1055 is a powerful inhibitor of histamine formation by mast cell both in vitr and in vivo

According to Rothschild and Schayer (1959) histamine formation in mast cells is brought about by a specific histidine decarboxylase which is not inhibited by a-methyldopa. We have shown that a-methyldopa will inhibit the formation of histamine by whole mast cells in vitro presumably this is due to inhibition of histidine uptake rather than histidine decarboxylation.

Moran, U näs and Westerholm (1962) found the mean histamine content of rat peritoneal mast cells to be 31.5 µg per million cells, while the mean 5HT content was only 1.34 µg per million cells. The presence of dopamine in normal rat peritoneal mast cells has not yet, to our knowledge, been demonstrated. Hagen *et al.* (1960) reported that in murine neoplastic mast cells, despite the presence of dopa decarboxylase, no dopamine was detected.

W in estigated the ability of normal rat mast cells to decarboxylate histidine, 5HTP and dopa in vit o Assuming that only the L-forms of the amino acids are decarboxylated, that no conversion from D to L form occurs in the mast cells and that the D forms do not interfere with decarboxylation we can say that decarboxy lation of 5HTP and dopa occurs more readily under our conditions, than that of histidine. Why then in normal rat mast cells is dopamine absent and the content of 5HT so low compared to that of histamine

A possible explanation is that the cells can synthesize all three substances but are unable to store them in a manner similar to that used for histamine (vid Thon and Uvnäs 1966) However this explanation seems unlikely since Bergendorff (personal communication) has demonstrated that vit isolated rat peritoneal mast cell granules ar able to take up and store both 5HT and dopamine. In addition Fu rano and Green (1964) ha e shown that normal mast cells are capable of retaining both histamine and 5HT

Lovenberg, Weissbach nd Udenfriend (1962) reported that 5HTP and dopa are not normally detectable in the tissues. We investigated the ability of normal mast cells to hydroxylate tryptophan, phenylalanine and tyrosine vitro and could not detect any such activity Day and Green (1962) showed that neoplastic murine mast cells could hydroxylate tryptophan but not tyrosine or phenylalanine. Using neoplastic cells originally derived from the same train of cells, Levine Lovenberg and Sjoerdsma (1964) showed that tryptophan and phenylalanine ould be hydroxylated, the latter to tyrosine. Sch ndler (1958) also reported th presence of tryptophan-5-hydroxylase in neoplastic m rine mast cells. However t does not yet ppear to ha e been shown that these ells can form the immediate precursor of dopamine dopa, from tyrosine

From our results it ppears that the comparativel low content of 5HT and the absence of dopamine n normal rat mast cells is probabl due to the una ailability of the immediate precursors (5HTP and dopa nd t the inabilit of th cells to synthesize them by h droxylation of tryptophan tyrosine respectiv ly

According to Diamant and Lowry (1966 the me n dry weight of rat r

mast cells is 476 pg. Assuming that normal mast cells contain about 80 % water the average wet weight would be approximately 2.5×10 g. From our results the histamine formation, with a substrate concentration of 10^{-3} M, was about 30 ng per million cells in 3 hrs. This is equivalent to 12 µg of histamine per g of mast cells (wet weight) in 3 hrs. Thus it would take about 18 weeks for 10^6 mast cells in a medium containing 10^{-3} M histidine to synthesize 30 µg of histamine, their normal content, if the synthesis continued at the same rate.

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A Comparative Study of Membrane Properties of Innervated and Chronically Denervated Fast and Slow Skeletal Muscles of the Rat

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Abstract

ALBUQUERQUE, E. X. and S THESLEFF *A comparative study of membrane properties of innervated and chronically denervated fast and slow skeletal muscles of the rat* Acta physiol. scand. 1968 73 471—480.

The electrophysiological properties of the innervated extensor and soleus muscles are in most aspects similar. In low $[Na]_o$, however the rate of rise and the amplitude of the action potential were lower in the soleus than in the extensor muscle. The ACh sensitivity in the innervated extensor muscle is limited to the end-plate region, while in the soleus the sensitivity extends beyond the end-plate towards the tendon. Denervation brought marked change in membrane properties. The electrical time constant of the membrane increased in both muscles almost threefold, and the calculated transverse resistance of unit area of the membrane in the extensor from 372 Ω cm² to 759 Ω cm² while in the soleus the values were 393 Ω cm² to 458 Ω cm². Denervation increased the threshold for action potential generation and reduced significantly the rate of rise and the amplitude of the spike. These changes were more apparent in the soleus. It is suggested that the decrease in excitability may reflect the lengthening of the time constant and possibly reduction in the number of membrane sites connected with the passive ionic transport. The ACh sensitivity of denervated muscles covered the entire membrane surface and was similar in both muscles.

Functionally the limb muscles of mammals can be differentiated into fast and slow contracting muscles (Denny-Brown 1929). In slow muscle such as the soleus, the contraction time is two to three times greater than that of the extensor digitorum longus (extensor) fast muscle (Buller, Eccles and Eccles 1960 a, b, Close 1964). Fast and slow muscles also differ in their metabolic function (Needham 1926, Drabota and Gutmann 1963, Gutmann 1966, Guth and Watson 1967). While slow muscles are composed mainly of fibres containing a high quantity of oxydative enzymes, fast muscles are a mixture of fibres which have mostly glycolytic enzymes (Henneman and Olson 1965, Stein and Padykula 196). Another difference occurs in the distribution of acetylcholine (ACh) sensitive sites. As shown by Miledi ...

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Zelená (1966) the ACh sensitive area in the soleus muscle of the rat extends from the end-plate to the whole fibre surface while the ACh sensitivity in a fast muscle is restricted to the part surrounding the end-plate. Further evidence for a differentiation of membrane properties in fast and slow muscles were given by Albuquerque and Thesleff (1967 a) These authors observed that innervated and chronically denervated extensors of the rat were more sensitive to the enzymic actions of phospholipase C than soleus muscles.

Since the membranes of fast and slow muscles appear to differ in a number of aspects but little qualitative and quantitative information exists regarding their respective electrophysiological properties, a comparative study was designed with the objective of analysing some of the membrane characteristics of these muscles in the rat. The following electrophysiological properties of the innervated and chronically denervated extensor and soleus muscles were investigated first, the resting membrane potential and the electric membrane constants, secondly the action potential generating mechanism and thirdly the ACh sensitivity

Methods

All the studies were made "in vitro" on the extensor and the soleus muscle of male rats weighing 200—250 g. The general experimental conditions, and the recording techniques used for determining the resting membrane potential and input resistance has been described in detail by Albuquerque and Thesleff (1967 a, b)

Electric membrane constants were determined by applying the cable theory of Hodgkin and Rushton (1946) In one series of experiments on single fibres of the innervated extensor and soleus muscles the method of square pulse analysis of Fatt and Katz (1951) was used. With this method, the potential change (V) produced by steady current (I) through the muscle membrane is expressed as

$$V = 1/2\ I \sqrt{(r_m r_i)} \exp[-x\sqrt{(r_i/r_m)}],$$

[illegible] is the electrode separation, r_m the transverse resistance of unit length of fibre membrane [illegible] the internal longitudinal resistance per unit length of fibre.

[illegible] following technique was used to obtain the data A rectangular hyperpolarizing pulse of [illegible] 100 msec duration was passed through an intracellular microelectrode [illegible] initial distance of 0.05 to 0.1 mm from the recording [illegible]tracellular electrode. Subsequently the current passing electrode was withdrawn and reinserted at 0.3—0.5 and 0.8—1.0 and 1.3 mm distances Fibres which during this procedure depolarized by more than 5 mV were rejected The ratio V/I was calculated from the average of 3 different current pulses producing membrane potential change of 10—30 mV Only if the plot of these data, on logarithmic scale in the ordinate and the distance between the electrodes in mm on the abscissa, showed linear relation where the data accepted The space constant (λ) of the membrane was obtained directly from the slope of the straight line and the input resistance (R_{in}) was the point at the ordinate of abscissa zero Thus, the calculation of the transverse (r_m) and the internal longitudinal (r_i) resistances could be made The transverse resistance of unit area of membrane (R_m) was calculated from the equation $R_m = 2\pi\rho r_m$. The calculated fibre radius (ρ) was obtained from the equation $\rho = \sqrt[3]{(R_i/\pi r_i)}$ in which R_i is the resistivity of myoplasm. The specific resistance of the myoplasm at 25 °C was calculated from $R_i = \rho^2 \pi r_i$ in which [illegible] was taken as the average of those obtained from the innervated muscles and an assumed ρ of 21 µ, which agrees well with the calculated and the measured values for ρ, see Table I. With these assumptions the myoplasm resistance was estimated to be 180 Ω cm. The time constant (τ_m) of the membrane was determined from the time taken for the anodal potential to rise to 83 % of its maximum steady phase with the two microelectrodes separated 0.1 mm (Hodgkin and Rushton 1946) and the membrane capacitance (C_m) per unit area from $C_m = \tau_m / R_m$.

For measuring transverse fibre diameters in innervated and in chronically denervated extensor and soleus muscles were removed, and fixed in Bouin solution After imbedding in paraffine cross sections of 5—10 [illegible] thickness were made from the [illegible] part of the muscle

and stained according to an Gleson. The diameter of each muscle fibre was measured the aid of an ocular micrometer at 500 × magnification and expressed as the mean largest diameter and the diameter perpendicular to that one. I each muscle one h fibres ere measured.

For measuring the action potential tw microelectrodes were inserted into the same f with 0.05—0.1 mm spacing. One of the electrodes was used for current passing and the other for potential recording. In order to minimise the effect of ariations in the resting membrane potential the potential of the cell was locally set to lev l of 70—80 mV by passing constant current through the membrane, the current terminating in 30 msec cathodal shock adjusted in steps of increasing magnitude until an action potential was produced. Details of this procedure and the technique for measuring the action potential are gi en by Albuquerque and Thesleff (1967 b)

The ACh sensitivity of the muscle membrane was determined by micro-iontophoretic appli cation of the drug as described by del Castillo and Katz (1955) and Katz and Miledi (1964) The ACh pipette was f lled with 5 M ACh solution. The experimental procedure was similar to that described by Katz and Miledi (1964) and w adopted the same unit for ACh sensitivity i.e., the amplitude, in mV of the transient membrane depolarization due to the ACh pulse divided by the coulomb × 10 quantity passed through the ACh pipette (1 unit = 1 mV/10 C) The end-plate region of innervated muscles was localized by the recording of miniature end-plate potentials w th fast rising phase. When the distribution of ACh sensitivity along the length of the muscle fibre was studied, the ACh pipette and the recording electrode were moved together at intervals of 0.5—1.0 mm til the fibre tendon was reached. The spacing between the ACh pipette and the recording electrode was, in general, 0.05—0.2 mm. Fibres in hich the resting membrane potential fell by more than 15 mV were discarded.

The bath fluid had the composition described by Albuquerque and Thesleff (1967) I as oxygenated by bubbling with 95 % O_2 + 5 % CO_2. The pH of the solution was 7.0—7.3 and the bath temperature 23 C. When low external sodium chloride concentrations were used the osmolarity was kept by replacing the ions with sucrose.

Results

Resting membran potential and electric memb ane nstants The mean resting membrane potential of 345 surface fibres in 20 innervated extensor muscles was −72 ± 3.4 (S.D) mV and of 179 fibres in 15 innervated soleus muscles was −69 ± 5.5 mV Increasing the external potassium concentration $[K]_o$ from 5 to 15 mM reduced the resting membrane potential of the extensor and the soleus to −55 ± 2.9 mV (45 fibres) and −54 ± 2.7 mV (39 fibres) respectively

The resting membrane potential of chronically denervated muscles was by 10—15 mV lower than in innervated muscles. In the extensor the mean value of 264 fibres was −57 ± 5.3 mV and in 299 fibres of the soleus −55 ± 3 5 mV The dif ferences in membrane potential between innervated and chronically denervated muscles were highly significant (P<0.01) whil the differences between fast and slow muscle were not significant.

The membrane constants of 9 fibres in the innervated extensor nd of 10 in the innervated soleus muscle were calculated by the use of the square pulse analysis of Fatt and Katz (1951) The mean alues obtained are given in Table I

The equation $R_m = \pi^2 R_{in}^2 d^3 / R_i$ provides a way for calculat ng th transverse resistance of unit area of muscle membrane (R_m) without th use f multiple deter minations of the nput resistance in single fibres. Only the input resistance (R_{in}) and the fibre diameter had to be experimentally determined The myoplasm resistance (R_i) was again as med to be 180 Ωcm see Methods The alculated fibre space constant was obtained from the expression

$$\lambda = 2\pi \rho^2 R_{in}/R$$

TABLE I Electric membrane constants of muscle fibres in innervated and 7–10 days denervated extensor and soleus muscles at 23 °C. RMP resting membrane potential; R_{in} input resistance; λ, fibre space constant; τm membrane time constant; ρ fibres radius; R_m transverse resistance of unit area of membrane, and C_m, membrane capacitance per unit area

Muscle	RMP mV	R_{in} MΩ	λ mm	τm msec	ρ μ	Rm Ω cm	Cm μF/cm
		Innervated					
Extensor[1]	72 ± 1.7 (9)	0.39 ± 0.06	0.54 ± 0.05	1.7 ± 0.1	21 ± 2.1	559 ± 72	3.3 ± 0.4
Extensor		0.40 ± 0.16 (67)	0.4		17[a]	372	4.5
Soleus[1]	71 ± 2.0 (10)	0.32 ± 0.05	0.56 ± 0.05	1.7 ± 1.0	24 ± 1.9	485 ± 47	3.5 ± 0.2
Soleus		0.44 ± 0.14 (79)	0.4		17[a]	393	4.3
		Denervated					
Extensor		1.11 ± 0.36 (79)	0.5	4.4 ± 1.3 (27)	12[a]	759	5.8
Soleus		0.76 ± 0.19 (77)	0.4	4.8 ± 1.7 (29)	12	4.6	10.8

Results obtained in single fibres by the use of the method of square pulse analysis (Fatt and Katz 1951)

Mean ± S.D. the figures in parentheses show the number of fibres analysed.
a—Mean of 500 fibres.

As shown in Table I the values for membrane constants obtained by the two techniques are very similar and it can be inferred that the membrane constants of the innervated extensor and soleus muscles are essentially identical. Following denervation, however, the membrane resistance of the extensor is nearly doubled while that of the soleus remained almost unchanged. The time constants of both muscles were markedly increased by denervation from 1.7 to 4.4 and 4.8 msec respectively as were the capacitances, particularly in the soleus muscle.

Action potential The shape of action potentials in the innervated extensor and soleus muscles were essentially similar but the duration slightly prolonged in the soleus as shown by the records in Fig. 1 A. The mean values for the amplitude of action potential, its rate of rise and the threshold potential for its generation for both muscles are shown in Table II.

Since the rate of rise and the peak amplitude of the action potential are related to [Na] (Katz 1947; Hodgkin and Katz 1949) experiments were made in which the muscles were allowed to equilibrate for 1 hr in a solution containing 45 mM NaCl instead of the normal 135 mM, osmolarity being kept by the addition of

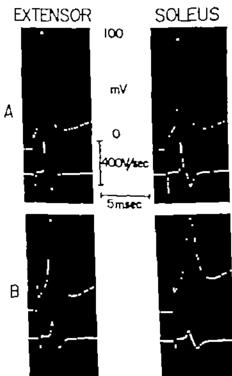


Fig. 1

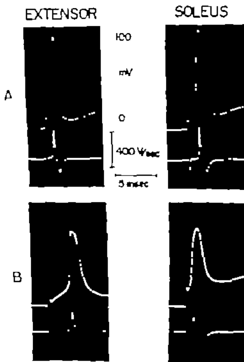


Fig. 2

Fig. 1 Typical records of the effects of low [N]e on action potential generation in innerv ted extensor and soleus fibres. A, illustrates the intracellularly recorded spike and it first deriv t in 135 mM NaCl and B, in 45 mM NaCl

Fig. 2. Intracellular records from the tensor and soleus muscles showing the changes in membrane excitability caused by chronic denervation. A, shows the action potential and ts f rst derivati in innervated muscles and B, in muscles denerv ted 7—10 days.

sucrose. This reduction in $[Na]_e$ had a negligible effect on the resting membrane potential, which fell by about 2 mV As shown by the mean values Table II the threshold potential for the spike increased in both muscles by about 50 per cent while the spike ampl tud fell by 25 per cent in the extensor and by 40 pe cent in the soleus. The most marked hange occurred in the rate of rise of the action potential which was reduced from 336 to 139 V/sec in the extenso and from 296 to 70 V/sec in the soleus. This difference between the two muscles in the rate of rise of the spike in low [N] was significant ($p<0.01$) Typical records of action potentials n respectively 135 and 45 mM $[Na]_e$ are shown in F g 1

Action potential generation was also studied in preparations denervated for 7—10 days. This period of denerv tion caused a decrease in the membrane excitability as exemplified by the records in Fig. 2B. The mean values obtained for threshold potentials, amplitudes and the rates of rise of the action potentials in the two muscles are given T ble II. I both muscles the threshold for excitation are

TABLE II Threshold, amplitude and rate of rise of action potentials in innervated and chronically denervated extensor and soleus muscles. The values are the means ± S.D. and the figures in parenthesis show the number of fibres analysed

Muscle	$[Na]_o$ mM	Threshold mV	Amplitude mV	Rate of rise V/sec
		Innervated		
Extensor	135	23 ± 7.0 (242)	89 ± 14.6	336 ± 35.3 (181)
Soleus	135	22 ± 7.6 (66)	91 ± 12.9	290 ± 35.6 (66)
Extensor	45	34 ± 3.3 (34)	67 ± 4.6	139 ± 33.8 (34)
Soleus	45	37 ± 4.6 (32)	53 ± 8.3	70 ± 28.4 (32)
		Denervated		
Extensor	135	26 ± 7.4 (36)	69 ± 10.8	202 ± 78.0 (36)
Soleus	135	34 ± 16.4 (46)	74 ± 19.0	123 ± 60.4 (32)

many fibres, particularly of the soleus muscle, spikes were not generated. The amplitude of the action potential decreased by about 20 per cent in both the extensor and the soleus and its rate of rise by 40 and 58 per cent respectively. These reduc[illegible] in excitability are significant ($P = 0.01$) but not the differences between ex[illegible]r and soleus.

ACh s[illegible]sitivity

The ACh sensitivity of the muscle membrane was determined in the innervated and chronically denervated extensor and soleus muscles. In the extensor and the soleus the end-plate is located in the center of the muscle fibre and in innervated fibres the recording of miniature end-plate potentials with a fast rising phase showed the exact location of the end plate. In 24 fibres of 5 innervated extensor muscles and in 33 fibres of 7 innervated soleus muscles the mean ACh sensitivity of the end-plate region was 107 and 92 units respectively. The highest spot sensitivity to ACh recorded within that region was 230 units in the extensor and 180 in the soleus. The rise time of these potentials were respectively 3.4 and 4.2 msec. An ACh potential at a particularly sensitive spot in the extensor muscle is illustrated in Fig. 3A. Moving the ACh pipette 200μ away from the end-plate reduced the ACh sensitivity of the membrane to a low value compared to that observed focally (Fig. 4). In the extensor a further shift of the pipette to 300μ almost completely abolished ACh sensitivity (10 units) and at 400μ no response to ACh was recorded. In contrast to the extensor the membrane in the soleus was sensitive to ACh outside the

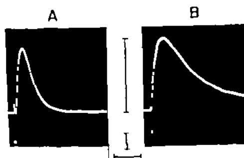


Fig. 3. Acetylcholine potentials evoked by micro-iontophoretic application of the drug to "high sensitivity spots" in the innervated (A) and chronically denervated (B) extensor muscle. In A, the ACh-pipette was at sensitive spot in the end-plate region and in B in the center of denervated muscle fibre.

Calibrations: The upper vertical line represents the voltage scale, A = 11.5 mV and B = 5.0 mV and the lower vertical line the coulomb number A = 10.5 × 10 A and B = 5.2 × 10 A. The duration of the current pulse delivering ACh was in both records 1 msec. Time scale for A = 11.5 msec and B = 20.0 msec. Resting membrane potential was for A and B respectively 73 mV and 61 mV.

end-plate region. A sensitivity of about 10 units was observed several mm away from the end-plate and in about 65 per cent of the soleus fibres the entire surface had a low sensitivity (10 units) to ACh (Fig. 4). As is further shown by the graph in Fig. 4 the ACh sensitivity in the soleus again increased towards the tendon region where it was as high as one hundredth of that at the end-plate, i.e. about 1 unit. No response to ACh was recorded at the tendon regions of extensor muscles.

Following denervation the entire muscle membrane became highly sensitive to applied ACh as has previously been described by Axelsson and Thesleff (1959). In a period of 7—10 days following sectioning of the motor nerve any spot in the membrane was sensitive to ACh. The mean sensitivity in the extensor and in the soleus was 51 (90 fibres) and 54 units (95 fibres) respectively. Occasionally spots with a higher ACh sensitivity were recorded in the centre of denervated muscle fibres (Fig. 3B). The highest "spot" sensitivity recorded was 80 units with a rise

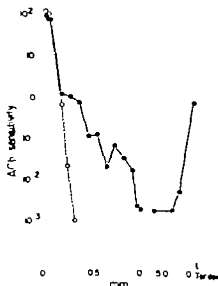


Fig. 4. Distribution of the ACh sensitive membrane area in single fibres of the innervated extensor (open circles) and soleus (closed circles) muscles. Each point represents the mean of measurements in 4 to 15 fibres. Abscissa, distance in mm from the end-plate along the fibre; ordinate: the log. for ACh sensitivity.

time of about 10 msec. In comparison with a high sensitivity spot at an innervated end plate the rise time and the duration of ACh potential was markedly prolonged as shown in Fig. 3 The explanation for this is presumably the lengthening of the time constant of the membrane that was observed following denervation and the lack of cholinesterase in regions outside the end plate.

Discussion

The present study has shown that the electrophysiological properties of innervated extensor and soleus muscles are in most respects similar The only differences observed were in the presence of low $[Na]_o$ when the rate of rise and the amplitude of the action potential in the soleus were lower than in the extensor Furthermore, we confirmed the observation of Miledi and Zelená (1966) that the distribution of the ACh sensitive area in the two muscles are different. In the extensor only the end plate region was sensitive to applied ACh while in the soleus the ACh sensitivity extended beyond the end-plate and in a majority of the fibres the entire membrane had a low sensitivity to the drug

Following denervation the membrane properties of both muscles markedly changed. The input resistance and the electric time constant of the membrane were significantly increased. the time constant was almost three times its value in an innervated muscle The calculated transverse resistance of unit area of membrane had almost doubled in the extensor but was little changed in the soleus. Thus, the membrane capacitance increased more in the chronically denervated soleus than in the extensor muscle since both muscles had identical time constants.

The observed changes in the electrical properties of the membrane apparently [illegible]ct structural alterations in the cell membrane since it is unlikely that the re[illegible]nce of the myoplasm had markedly changed. The increase in membrane [illegible]tance could be the result of a reduction in the potassium conductance of the membrane as has been postulated by Klaus, Lüllmann and Muscholl (1960) for the chronically denervated rat diaphragm. The reduction of potassium conductance would enhance the influence of sodium and chloride ions on the resting membrane potential and thereby explain the lower membrane potential of denervated muscle (Lüllmann 1960 Thesleff 1963)

The studies of Falk and F[illegible]tt (1964) indicate that muscle fibres have two capacitative channels which charge at different rates. The surface membrane represents a smaller value while the sarcoplasmatic reticulum provides a larger capacitance The observed increase in membrane capacitance and the prolongation of the time constant may reflect post denervation change in the structure of one or both of these elements

According to the ionic hypothesis the rate of rise of the action potential is the result of a high, selective and brief movement of sodium ions across the membrane (Hodgkin and Katz 1949 Differences in excitability as was revealed in the presence of low $[Na]_o$ between the innervated soleus and the extensor presumably

reflect a quantitative difference in the sodium carrying mechanism of the membranes of these two muscles.

Denervation increased the threshold for action potential generation and reduced significantly the rate of rise and the amplitude of the spike the changes being most marked in the soleus. The observed decrease in excitability following denervation may reflect a reduction in the number of membrane sites connected with passive ionic transport. On the other hand, a low resting membrane potential and a lengthening of the electric time constant of the membrane, as was observed in denervated muscles, would, in themsel es, be inhibitory to action potential generation.

Despite a marked difference in the distribution of ACh sensitive sites among the two innervated muscles, chronic denervation led to identical changes in the extensor and the soleus. Both muscles developed a high and uniform ACh sensitivity which covered the entire cell surface. A consistent finding was that the pparent highest spot sensitivity of a denervated membrane was less than that of an innervated end-plate. This observation might indicate a true difference in receptor sensitivity bet een the two conditions, the ACh receptors of the innervated end plate being more sensitive than those of the chronically denervated membrane. On the other hand, the observed lengthening of the electric time constant of the denervated membrane is in itself a sufficient explanation for the difference in sensitivity to ACh applied iontophoretically by a current pulse of only a few msec duration (see Fig. 3)

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Induction and Inhibition of Pinocytosis in Amoeba Proteus

By

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Abstract

Josefsson, J.-O. *Ind ction and inhibition f pi ocytosis in Am eba pr teus.* Acta physiol. scand. 1968. *73* 481—490.

Pinocytosis induced by different cations was studied by phase contrast in *Amoeba proteus.* Monovalent cations were more effectiv inducers the smaller the hydrated size of the ion (Cs>K>N >Li). Divalent cations were less activ inducers than monovalent ions and among the divalent ions tested alcium was unable to induce measurable pinocytosis. Tris possessed properties as an inducer similar to sodium although the intensity of Tris-induced pinocytosis s greater. The sensitivity to the inducing cation decreased in proportion to the concentrations of calcium and hydrogen ions in the medium. Addition of calcium chelating agent to the inducer decreased the maximum numbe of channels observed. Among the divalent cations present in the culture solution only calcium could restore the pinocytosis intensity to normal. The receptors in the cell membrane upon which inducing ions act are proposed to be negatively charged groups, the ionization of which aries with pH. Their availability to inducers diminished when the calcium concentration of the medium is increased. A minimal calcium concentration is, however, required for normal function of the channel forming system. The difference in activity between potassium and sodium in the induction of pinocytosis might be factor controlling the intracellular content of these ions in the amoeba. The outward diffusion of potassium from the cell migh explain the phenomenon of spontaneous pinocytosis.

Pinocytosis, a word coined by Lewis (1931) to describe the morphological picture of uptake of fluid droplets by macrophages, has been observed in several species of amoebae by Mast and Doyl (1934). The mechanism of pinocytosis is still unknown but its details can be separated into two phases. The first phase is the adsorption of the inducer to the mucous coat of the membrane. The second phase is the formation of pinocytosis channels, which unlike th first is temperature dependent and inhibited by metabolic blockade (Schumaker 1958, de Terra and Rustad 1959). Several organic and inorganic cations are effective as inducers (Chapman-Andresen 1958, Rustad 1959). These cations also ncrease the ionic permeabil ty of the membrane and depolarize the cell, whil mono ar without these properties (Josefsson 1966). The bioelectric effects re ggested to result from the binding of the inducer to fixed anionic groups in th mucous coat of the amoeba. Whether a physiologically important interplay bet een these qualities and pinocytosis exists is not known. In this investigation mbe of inorganic cations, which were thought

to differ in their effect on membrane permeability were tested as inducers or as inhibitors of pinocytosis. As a pinocytosis inducer the organic cation Tris (tris-hydroxymethylamino methane) possessed properties similar to sodium and was used to replace the latter in some experiments. Effects of hydrogen and calcium ions on the minimum effective concentration (affinity) of the inducer to adsorb to the cell membrane are reported as well as the effect of calcium on the activity (ability to induce channels) of different inducers.

Methods

Stock cultures of *Amoeba proteus* were fed on Tetrahymena according to the technique of Prescott as modified by Chapman-Andresen (1958). The culture medium (Pringsheim) had the following composition (mM): Na^{+} 0.22, K^{+} 0.35, Ca^{+2} 0.85, Mg^{+2} 0.05, F^{+3} 0.007, $HPO_4^{-2}+H_2PO_4^{-}$ 0.11, Cl^{-} 0.35, NO_3^{-} 1.70, SO_4^{-2} 0.09.

Microscopic observations were made on living amoebae using a Leitz phase contrast system with 600 × magnification. The microscope stage was used in the horizontal position. All experiments were made at a room temperature of 22 °C. The temperature at the microscope stage was not controlled in each experiment but marked temperature changes were excluded by prewarming the stage and by keeping the current setting of the transformer at a constant value. All salts used in the experiments reported here were chlorides of analytical reagent grade. Alcian blue was obtained from George T. Gurr Ltd, London, England and EGTA (1,2-Bis-2-aminoethoxyethane-tetraacetic acid) from Hopkins and Williams Ltd, Chadwell Heath, England. Tris (tris-hydroxymethylamino methane) was a product (Lot nr 103B-5700) of Sigma Co, St. Louis, USA. In order to avoid calcium-binding effect of the common buffer systems adjustment of pH of the salt solutions were made by addition of HCl or the hydroxides of the cation under investigation. Measurements of pH was made in the solutions before and after the experimental period (20 min). Spontaneous drift exceeding 0. pH-unit during this period was not allowed. All values of pH given in the following refer to the initial pH.

The amoebae were taken from cultures starved for 3 days, carefully washed with Pringsheim solution, and placed on slides in a moist chamber for 30 min before the experiment to allow the cells to attach to the glass surface. At the end of this period the slide was tilted about 30 degrees to the horizontal plane and washed with 2—3 ml of Pringsheim in order to remove those cells which had not attached. At zero time the Pringsheim solution was removed with ... it and replaced by the test solution. This exchange procedure was repeated three times ... olume of the inducer was 1.5 ml and the time required for this manoeuver was 1 min. ... est solution was covered by ... overslip and the counting procedure started. For the purpose of determining the relative efficacy of inducers as well as the effect of alcium-enriched and alcium-deficient media the following method, a simplification of the technique described by Chapman-Andresen (1962) was regarded to be adequate. Each amoeba was observed for 1 min and the number of pinocytosis channels present, developing or ... during that time was noted. By using the ... of the coordinate system of the stage it was possible to ensure that no amoeba was observed twice. The duration of each experiment was 20 min. The sum of the number of channels observed in this way (from the beginning of the 2nd to the end of the 20th minute) was divided by the number of cells (19) and the resulting mean number of channels per amoeba was used to express the intensity of pinocytosis. Only a ... estimate could be made as an unknown fraction of the channels were not visible from the horizontal position. Those salts which are stated to be ineffective as inducers might therefore with a more sensitive method, reveal some inducing capacity. Because of the limited time available for counting, samples in very intense pinocytosis (mean number of channels observed per amoeba exceeding ten) were probably underestimated as compared to those in moderate or pinocytosis. Due to the shorter life time of the individual channels observed in minimal pi... than these values might also be too low.

The time distribution of channels showed a maximum between 10 and 20 min ... with all inducers examined except when EGTA was added to the inducer in order to bind calcium ions (Fig. 7). In that instance most of the channels developed early in the cycle of pinocytosis, and therefore gave relatively high values for pinocytosis.

As previously remarked by Chapman-Andresen, results obtained with amoebae from one culture dish during one day were more consistent than results obtained from different cultures and from different days.

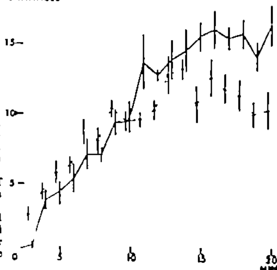


Fig. 1 Pinocytosis induced by 40 mM KCl pH 5.7 Ordinate: Pinocytosis intensity, i.e. number of channels observed per cell. Abscissa: Minute in the pinocytosis cycle. Each point in the graph indicate the mean number of channels observed in 11 cells during respective minute. Bars indicate standard errors of the mean. Solid line and dots: 11 groups of 19 amoebae, each group taken from separate subculture. Broken lines and triangles: 11 groups of 19 amoebae all groups taken from one subculture. For clarity in the figure the time scales for the curves are separated.

At eleven different occasions during a period of six months the response to potassium 40 mM KCl gave a pinocytosis index of 10.3 ± 0.44 (n = 11). When the measurements were completed in 8 hrs using cells from one subculture the result was 8.9 ± 0.21 (n = 11). Fig. 1 gives the mean number of channels observed each min in the aforementioned two series of experiments. The curves drawn through the means of the number of channels indicate the onset and development of pinocytosis during the first 20 min of the cycle.

The phenomenon of pinocytosis in addition to the adsorption phase also includes channel formation, each of these phases could be pH sensitive. Results with uranyl ion or alcian blue as inducers (to be reported elsewhere) indicate that the hydrogen ion concentration determined the number of channels only during the period of adsorbtion of the inducer to the membrane. The processes responsible for morphological changes were less dependent on the hydrogen ion concentration.

Results

Dose-response curves of different inducers

Plots of the numbers of channels in different concentrations of the chlorides of cesium, potassium, Tris, sodium and magnesium are given in Fig. 2. The curves were obtained in experiments on different days with amoebae from different cultures but their mutual relations have proved to be constant. In the figure the values for Tris and magnesium were obtained at pH 7.0 while the sodium potassium and cesium solutions were at a pH of 5.7—5.8. Tris buffer was used with Tris as the protonated cation and with chloride as the anion. Cesium and potassium at pH 5.7 were maximally inducing at 35 to 40 mM. At higher concentrations the response became gradually smaller. If the maximal number of channels is taken as a measure of the activity of the inducer the activities of cesium and Tris were high while those of lithium and the divalent ions (magnesium, barium and strontium) were low. In calcium (10—200 mM at pH 5—7) practically no channels were seen, confirming the observation of Chapman-Andresen (1958).

If the lowest concentration capable of inducing pinocytosis was regarded as a

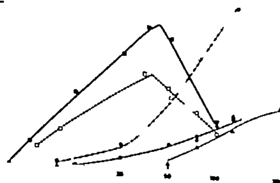


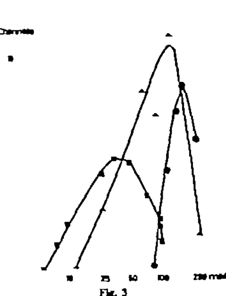
Fig. 2. Dose response curves for different inducers. Symbols in the figure ■—■ CaCl, □- -□ KCl, ○ ○ Tris-Cl, ● ● NaCl, ▲ ▲ MgCl. The channels were counted at about pH 5.7 except for Tris and magnesium which were at pH 7.0. The sodium curve is drawn through the means of the values for 2—6 expts. at each concentration. All other points refer to single experiments.

measure of the sensitivity of the mechanism of pinocytosis to the respective inducer the monovalent cations could be arranged in two groups. In one were cesium, potassium and rubidium, to which the membrane was highly sensitive. In the second were the inducers of weak affinity, namely Tris, sodium and lithium. The ions of the alkaline earth metals were similar to the latter group. The addition of sucrose 100 mM to the inducers did not alter the number of channels observed in Tris 75 mM, while the sensitivity to potassium was slightly increased, 5 mM KCl+ sucrose being equipotent to 10 mM without sucrose added.

Effects of calcium ions on pinocytosis

When calcium was added to the inducing solutions the respective dose-response curves were shifted to the right, i.e. the sensitivity to the inducers diminished. Fig. 3 gives the results of experiments with 0.2 and 3.2 mM $CaCl_2$ added at different potassium concentrations. The maximum of the reference curve was shifted from about 40 mM to 100 mM with 0.2 mM of calcium and to 200 mM by 3.2 mM. When still higher calcium concentrations were used the potassium curve was displaced further to the right and the intensity of pinocytosis subsequently decreased. To abolish the channels in 100 mM potassium the presence of 10 mM calcium was required. None of the other alkaline earth metals did produce a similar sensitivity decrease. The mutual potency of some divalent ions to inhibit the pinocytosis was Ca > Mn > Ba > Sr > Mg.

Through its competitive like inhibitory effect, a continuously elevated calcium level should, at any potassium concentration beyond 40 mM, increase the number of channels to a maximum and subsequently inhibit pinocytosis. A curve as simple as this was only obtained at pH (Fig. 4). At pH 5.7 the first part of the curve was biphasic or polyphasic and at pH 4.0 to 4.5 no potentiation of pinocytosis was observed. The increase noted in the number of channels was not a specific effect of calcium. Curves similar to those given for calcium and magnesium (Fig. 4) at pH 5.5 were also obtained with barium, strontium and managanese ions, although ferrous and zinc ions at pH 5.7 exhibited no biphasic action.



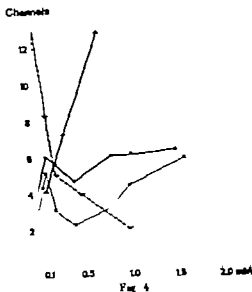


Fig. 3

Fig. 4

Fig. 3. Effect of calcium addition on the intensity of pinocytosis produced by potassium at pH 5.7. The symbols in the figure denote KCl (■), KCl + 0.2 mM $CaCl_2$ (▲) and KCl + 5.2 mM $CaCl_2$ (●).

Fig. 4. Potentiation of pinocytosis, elicited by 100 mM KCl by addition of calcium and magnesium ions. The effect of calcium was tested at three different pH:s. The symbols denote KCl + $CaCl_2$ at pH 7.0 (▲), pH 5.5 (●) pH 4.0 (▼) and KCl + $MgCl_2$ pH 5.7 (■). Experiments indicated by different lines were made on cells from different subcultures. The inhibitory effect of some calcium concentrations at pH 5.5 was general finding although the exact position of the minima of the curves varied.

Calcium ions in concentrations of 0.1 to 0.2 mM added to Tris, sodium and magnesium always decreased pinocytosis induced by any concentration of these ions within 10—150 mM. The dose-response curves were, as with potassium, shifted to the right, indicating a diminished sensitivity of the receptor sites. When the cell culture was pretreated 1—3 hrs with 10—30 mM calcium, the normal pinocytosis inducing activity of Tris was not obtained despite extensive washing with Pringsheim before the change to Tris solution. Pinocytosis could, however be restored to a certain extent by an increase in the concentration of the inducer. The failure to reach the previous intensity was probably due to the osmotic strain exerted by these concentrations causing disruption of some of the cells. Attempts to find other inhibitors of pinocytosis as effective as calcium were unsuccessful. Barium, manganese, cobalt and strontium in this order at a concentration of 1 mM decreased the number of channels found in the presence of 100 mM or 125 mM of Tris (pH 6.5) by 90—75 per cent. Magnesium in 1 mM concentration inhibited this pinocytosis only by 50 per cent, while calcium produced almost 100 per cent blockade. Pinocytosis induced by the basic dyes acridin orange and alcian blue was inhibited by calcium to a lesser degree than that elicited by monovalent cations. The pinocytosis caused by acridin orange (0.1 mg/ml) was inhibited 50 ... 1 mM

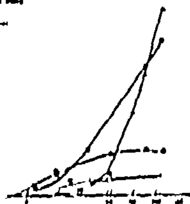


Fig. 5

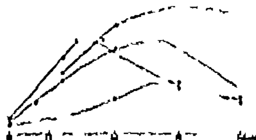


Fig. 6

Table I. Reversal of EGTA-inhibited pinocytosis by addition of divalent ions as per the pinocytosis intensity observed in the presence of Tris-Cl 1.5 mM and KCl at pH 7.0

Inducer (Conc. in mM)		Pinocytosis intensity (In per cent
Tris 12,5		100
12,5+EGTA 2,0		10
12,5+	2,0+Ca 0,5	30
12,5+	2,0+Ca 1,0	93
12,5+	2,0+Ca 2,0	
12,5+	2,0+Mg 0,1–0,5	15–20
12,5+	2,0+F 0,1–0,5	15–20
KCl 25		100
25+EGTA 2,5		25
25+	2,5+Ca 1,5	140
25+	2,5+Ca 2,0	170
25+	2,5+Ca 2,5	20
25+	2,5+Mg 0,1–1,0	20
25+	2,5+F 0,1–1,0	50
25	2,5+Mg 0,1	150
25	2,5+F 0,1	140

pared with the one observed for inducers of weak affinity (Tris and sodium). Thus like the effect of calcium on pinocytosis developed in high potassium solutions, EGTA exhibited both a negative and a positive influence on this process. As illustrated in Table I, the addition of calcium to KCl+EGTA increased the number of channels formed to values above the normal for this inducer with no calcium buffer system added. The calculated concentration of free calcium that allowed intensive pinocytosis to occur was in these and similar experiments within the range of 0.2–1.0 µM. Log. K_{Ca} for EGTA at pH 7.0 was taken to be 6.6 with no corrections for differences in ionic strength of the inducers.

The inhibitory action of EGTA was always preceded by a short period of stimulated pinocytosis (Fig. 7). Inhibition was generally visible at the fifth minute. If introduced after a period of pinocytosis, EGTA did not diminish the formation of channels until ten minutes later when a pronounced decrease in number occurred. Thus calcium ions were either not necessary for developing channels or appeared to be less available to the chelator when the pinocytosis had started. This phenomenon was illustrated by the use of EGTA as shown in Fig. 7. There was generally a slight increase of pinocytosis when an ordinary pinocytosis cycle was interrupted and the preparation washed with fresh inducer.

The effect of pH on pinocytosis

The amoebae displayed increasing sensitivity to all inducers tested with decreasing hydrogen ion concentration. With Tris as inducer the pH optimum fo



Fig. 7 Histogram of the number of kanneds developed per amoeba and minute plotted as the means for periods of 5 or 4 (the first period) min. The broken lines indicate experiments in which new solution was added during the 14th min. Three different pinocytosis cycles are demonstrated (A, B and C) A and B was induced by KCl 60 mM. During the 14th min this solution was exchanged for KCl 60 mM (A) or KCl 60 mM+ +EGTA 20 mM (B) C represents the pinocytosis during an uninterrupted 30 min cycle in KCl 60+20 mM EGTA. All experiments were made at pH 7.0. Each curve give the mean of the results of 3 expts.

activity was about 7 0 (Fig 8) The fraction of this amine in cationic form is approximately 90 per cent at pH 7 1 and 80 per cent at pH 7.5 The smaller number of channels in this part of the curve may therefore to some extent be due to the diminished availability of cations. However in alkaline media above pH 7.5 cytolysis was observed and this might have influenced the shape of the curve at these values of pH. Maximal pinocytosis at neutral pH was noticed with all inducers of weak affinity (Tris, Na, Li and Mg)

The influence of pH on the sensitivity to inducers of strong affinity was somewhat different. The maximum of the potassium curve was shifted from 40 mM at 5 7 (Fig 2) towards 30 mM at pH 7 0 without any appreciable increase of

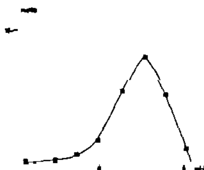


Fig. 8

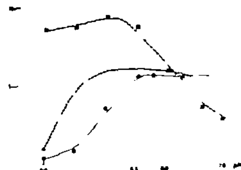


Fig. 9

Fig. 8. Effect of hydrogen ion concentration on pinocytosis induced by Tris buffer 125 mM (pK_a 8.1)

Fig. 9. Effect of hydrogen ion concentration on pinocytosis induced by potassium chloride at 20 (●), 37.5 (▲) and 100 mM (■). Experiments on three different subcultures were used to make the graph. The one used for 100 mM exhibited the highest pinocytosis intensity

pinocytosis intensity In acid conditions the maximum was moved to the right the abscissa towards 100 mM and simultaneously an increase in activity was m Thus the optimum pH for potassium was dependent upon the concentration of t inducer (Fig. 9)

Discussion

From the experiments of Chapman-Andresen (1958) (1962) it was ev dent that the ability of cations to induce pinocytosis was dependent upon the pH of the inducing solution. In the present investigation the sensiti ity of the cell to the inducing property of inorganic cations was found to increase towards neutral pH. As pinocytosis could be induced throughout a wide pH range one might suggest that several kinds of proton-accepting group of different pK are situated in the plasmalemma. These might, when associated with the ppropriat counterion, start the process of pinocytosis. The affinity of a monovalent cation for these receptors was greater the smaller the size of the hydrated ion. Thus a graded affinity in the sequence Cs>Rb=K>Na>Li was obtained.

It is interesting to not that the curve for potassium reached its peak at a concentration coinciding with the intracellular concentration of this ion. In the aforementioned series of cations, the depolarizing effect of the ions decreases from the left to the right (Josefsson, unpublished) The Input resistance" was earlier shown to decrease when inducers were applied to the membrane (Josefsson 1966) and recent ly Brandt nd Freeman (1967) described the increase of this resistance when the pinocytosis was inhibited by calcium. It therefore appears that there is a close connection between bioelectricity and pinocytosis. However until f rther knowledge of membrane potentials during pinocytosis is accumulated, the concept of pinocytosis will be discussed in terms of specific cation-sensiti receptors rather than of different polarized states of the membrane

The strong sensitivity to potassium relativ to sodi m might imply physiological role for potassium in induced pinocytosis. The permanent pinocytosis recently observed in Amoeba proteus (Stockem 1966, Wolfarth-Bottermann 1966) might be due to potassium ions accumulated in the mucous coat of the rod following ts diffusion from the i terior of the cell. Such a mechanism would serv to maintain the intracellular content (about 35 mM) of this ion.

The effect of calcium ions n pinocytosis could be explained by competition w th the inducing cation for receptors of pinocytosis and or for ligand which are not directly engaged in this process Calcium ions possessed in themsel e no pinocytosis inducing acti ty within 10—100 mM at neutral or acid pH Like h drogen ions they might affect the adsorption phase of pinocytosis by controlling th number of membrane sites for the inducing ion. Calcium chelating s bs nces would be expected to increase the number of ligands ailable to the nducer and thereby to enhance pinocytosis. The differenc in pH-optimum for potassium and sodium (or Tris) was most likely due to association with different polar groups of the m brane and difference in the esponse to calcium probably reflected

these ions to compete with calcium for their respective receptors. The marked sensitivity decrease imparted to the cell by calcium compared to other alkaline earth metals probably reflects its physiologically important property of being a noninducing cation.

The autoinhibition of pinocytosis by potassium in concentrations greater than 40 mM was pronounced at neutral but of little or no importance at acid pH. It was counteracted by calcium as well as by several other divalent cations. By postulating the presence of anions in the membrane ionized at neutral pH and capable of inhibiting pinocytosis when associated with potassium ions, this phenomenon and the pH-dependence of potassium pinocytosis could be explained.

The activity of an inducer is certainly determined not only by its affinity for an optimally organized membrane surface but also by a channel forming mechanism which is somehow triggered by the interaction between the inducer and the fixed anions of the membrane. Effects of the cation on metabolism or the unknown link between the two phases of pinocytosis could therefore interfere with the intensity of pinocytosis. The fact that Tris, sodium and potassium pinocytosis all required a certain amount of calcium which could not be substituted by either of the two other divalent cations in the culture medium (Mg^{++} and Fe^{++}) is an indication of a key role for calcium in the pinocytosis mechanism. From the observations on pinocytosis in solutions to which calcium chelators were added, the calcium ion might be suggested to act as a link between the membrane and the channel forming system. The latter might be activated by calcium ions which were released from the inside of the membrane or which entered the cell through gates opened by the inducer when it became associated with membranebound anions.

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Oxygen Uptake during Pinocytosis in Amoeba Proteus

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Abstract

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The oxygen uptake of *Amoeba proteus* was measured by the Cartesian ampulla diver technique. The intensity of pinocytosis was determined by counting the number of channels developed during the first 20 min of the pinocytosis induced by potassium chloride. Addition of calcium ions was used to inhibit pinocytosis induced by Tris. The oxygen uptake of the amoeba in culture medium was found to be 2.5 ± 0.9 µl/hr/cell at 25° C. Transient increases in rate of oxygen uptake were observed with different salt solutions. The intensity of respiratory stimulation varied in proportion to the activity of the salt as a pinocytosis inducer. Inhibition of Tris-induced pinocytosis by calcium and potentiation of potassium-pinocytosis by lanthanum decreased and respectively increased the respiratory rate of the amoeba. The time course of the development of channels was similar to that of the stimulated oxygen uptake. From experiments with a calcium-binding agent (EGTA) it was concluded that respiratory stimulation secondary to the increase of the permeability of the cell-membrane during pinocytosis might add to the energy expenditure during the formation of channels. As with the pinocytosis phenomenon calcium exhibited a dual action on the stimulated oxygen uptake. A minimal concentration was necessary for both processes while addition of calcium inhibited respiratory stimulation as well as pinocytosis.

Pinocytosis or cell drinking was described early and suggested to be a normal mechanism for the active uptake of molecules to which the cell membrane was impermeable (Lewis 1931). Recent work by Chapman-Andresen and Holter (1964) has stressed that the material adsorbed to the cell surface rather than the extracellular medium as such is concentrated by the cell during this process. Pinocytosis is induced by cationic molecules, which probably adsorb to negatively charged groups on the outer surface of the cell membrane. This adsorbtion is commonly accompanied by a decrease of transmembrane potential and input resistance (Josefsson 1966). During a second phase the membrane invaginates and from the channels so formed vacuoles begin to pinch off. A cycle of pinocytosis is complete in about 30 min after which the cell is relatively resistant to inducers for a couple of hrs (Chapman-Andresen 1963). Pinocytosis was recently shown to be markedly dependent on the calcium concentration (Brandt and Freeman 1967). During the adsorbtion phase this ion, which lacks inducing properties, probably competes w

the inducing cation for sites in the membrane (Josefsson 1968) Furthermore, inhibition of the pinocytosis in low calcium conditions was suggested to indicate requirement of this ion during the second phase. Apart from this artificially induced pinocytosis, spontaneous and permanent pinocytosis has recently been observed in *Amoeba proteus* by Wohlfarth-Botterman and Stockem (1966)

During phagocytosis in polymorphonuclear leucocytes and monocytes from guinea pigs, Sbarra and Karnovsky (1959) have shown that during aerobic conditions, a more than 100 per cent increase in oxygen uptake takes place. The increase in metabolism is caused by a stimulation of the direct oxidative pathway for glucose metabolism and is not cytochrome-linked.

The relationship between pinocytosis and oxygen uptake has not been studied previously From the work by Schumaker (1958) de Terra and Rustad (1959) and Chapman-Andresen (1966, 1967) we know that the formation of pinocytosis channels is delayed by low temperature and inhibited by cyanide carbon monoxide and several other metabolic inhibitors.

The rate of oxygen ptake increased when inducers where applied to the cell, the onset and duration of the stimulated respiration being coincident with the period when channels were present. By making use of the property of calcium ions to inhibit pinocytosis, as described in a preceding paper and the potentiating effect of lanthanum ions reported here, the respiratory increase was shown to ary in parallel to the intensity of pinocytosis.

In the present in estigation the oxygen uptake of the resting amoeba and of the amoeba in induced pinocytosis was tudied. An attempt has been made to correlate oxygen uptake and the formation of pinocytosis channels, the number of which was varied by the use of different inducers.

Material and methods

al d Method

amoebae ells w re Tetrahymena fed according to the method of Chapman-Andresen (1958 Prior to study the cells were starved for three days. Before taken for the experiment, they were caref lly washed th Pringsheim solution, the composition of hich has been given earlier (Josefsson 1966 Determination of pinocytosis intensity was made t 20—22 C according to a technique described in preceding paper

The oxygen uptake was measu ed by the ampulla diver technique thoroughly described by Holter and Zeuthen (1966) The divers were made from Pyrex glass using tubes (i.d. 8 mm, o.d. 9.8 mm) from which smalle and thinwalled capillaries were made (d about 2 mm, o.d. 2.1 mm) F om these pillaries, using microflame div rs ould be prepared ith neck length of 0 6—1 0 m and tail length of 2 0—3.0 cm The gas olume in balanced control divers was 0.5 —2.0 µl Before the experiment, the interior of the diver as siliconized with 1 per cent silicone in ethanol and dried for 2—3 hrs at 120 C. The filling operations had to be done under stereomicroscope at 40× magnification. The divers re inserted into wider glass tube connected to the mouth by rubber tubing. Pringsheim solution as sucked through the diver into the tail and air as let into the neck and the ampulla. A single amoeba in Pringsheim solution was sucked into the middle of the neck so that t was prevented from contact with the menisci The diver was sealed with heated mixture of wax and resin, separated from the glass tube and balanced in 0.1—N NaOH at 25 C. Finally it as transferred to a flotation essel containing 0 1—N NaOH and connected to manometer in the bath. Significant readings could be made about 20—30 min after equilibrium pressure as first established. The bath was t temper tu of 25 C, control was made by Yellow Springs Instrument Model 72 proportional temperature controller The room was termostated at 19°



Fig. 1 a. Time course of O uptake measured with the cell in Pringsheim solution. At 20 min this as exchanged for KCN 1 mM+NaCl 125 mM and the subsequent decrease of the rate of oxygen uptake is indicated by the diminished slope. The rate of 100 mm burette corresponds to an O uptake of 8.6×10 μl.
b. Control experiment in which the cell at 24 min (ertical line) was reincubated with the culture solution.

In most experiments the divers were later refilled with the same cell in new medium. The divers were taken out of the flotation essel thoroughly washed outside with Pringsheim solution at 25 C and inserted into wider glass tube just as in the first filling. The neck seal as cautiously picked off under the microscope and the diver placed with the neck in droplet of the new medium. Pressure was pplied until the cell was at the end of the neck and the new medium was sucked in. This procedure was repeated 3—4 times after which the neck as sealed, the diver balanced and transferred to its flotation essel in the bath. The time from refilling the diver until it was possible to make significant readings was about 10—15 min. The pressure adjustments and readings were done by means of "burette manometers calibrated as described by Holter and Zeuthen (1966) Control experiments with Pringsheim solution but without any cell gav an apparent gas exchange ranging from 0.29×10 μl/hr (ptake) to 0.11×10 μl/hr (evolution) Experiments in which the cells w re exposed to KCN 10^{-3}+NaCl 125 mM (to induce pinocytosis and to make sure that the cyanide got into the cell) showed sharp decrease in oxygen uptake (Fig. 1) In some control experiments the refilling was performed with Alcian blue (0.1 g/ml) and the final concentration of the dy in the diver was measured at 610 mμ. Adsorbtion of the dye to the siliconized diver was negligible during this procedure. The efficacy of the exchange procedure as constantly found to be better than 75 per cent

I most experiments, after refilling the diver there was an initial period of 10—15 min ith an apparent high ptake of oxygen (Fig 1b) This generally occurred in Pringsheim solution but was less prominent with incubation in calcium-rich media. Careless handling of the cell during incubation as ell as reincubation gav high respiratory rates of long duration. Cells hich did not exhibit an asteroid or amoeboid ppearan in the diver were not used.

All chemicals except lanthanum hloride were nalytical reagent grade. Tris (tris-hydroxy methylaminomethane) was product of Sigma Co St Louis, Mo. USA EGTA (1,2-bis-2 amino-ethoxyethane tetraacetic acid) was obtained from Hopkins and Williams, Chadw ll Heath, Essex, England.

According to the description of the supplier Schuchard Munchen) the lanthanum chloride used had purity of 99.99 pe cent The water onten was taken as 7 H_2O per molecule. Due to the uncertainty in the (re he molar ties gi en might be somewhat too high.

Result

Oxygen ptak cell m drum

The rate of oxygen consumption of amoeb in Pringsheim sol tion aried among the cells. Each cell, however exhibited consta t respiratory rate for several hours I 50 moebae the oxygen uptake aried from 0 3 10 μl/hr to 7 1 10 μl/l w th a mean of 2.5×10 the standard error of the mean being 0.86×10 d h In the experiments of Fig 2a the te of oxygen consumption was determir d repeatedly during 20—30 min and then the Pringsheim solution was replac d

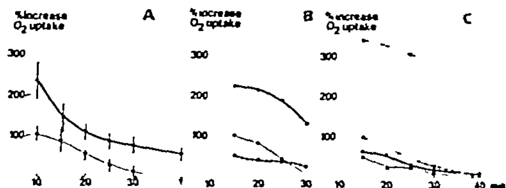


Fig. 2. Oxygen uptake in different salt solutions. Ordinate per cent increase of resting rate of O_2 uptake.
Abscissa. Time in minutes after incubation with the solution to be tested.
a. ○———○ Tris-SO 175 mM ○ ○ Pringsheim
b. ○———○ Tris-Cl 125 mM ○ ○ Tris-Cl 125 mM + $CaCl_2$ 1mM
○- ○ $CaCl_2$ 15 mM
c. ○ ○ KCl 175 mM + $LaCl_3$ 20 mM ○ ○ KCl 60 mM
○———○ KCl 175 mM ○ ○ Pringsheim

the same amount of new Pringsheim solution. After this reincubation the rate of oxygen uptake increased by 100 per cent, declining to the original steady-state level within 30 min (Table I)

Oxygen uptake during pinocytosis

The increase of the rate of oxygen uptake is given in the following as per cent of initial respiration measured in culture medium. Two types of inducers were used Tris (tris-hydroxymethylamino methane) and sodium both requiring a pH of 7.0 to give maximal pinocytosis, and potassium which is slightly more active at pH 4.5 in neutral solution. In the following experiments, Tris and sodium were always at optimal pH, while other inducers were at pH 5.7 As potentiating or m- g ions, calcium, magnesium and lanthanum were tested. Low calcium conditions were obtained by the use of the chelating agent EGTA (1 2-bis-2-amino-ethoxy)ethane tetracetic acid) The anion was generally chloride although sulphate ions were used in some experiments as they increased the pinocytosis evoked by Tris and sodium During the period of study (12 months) the amoebae apparently changed their sensitivity versus Tris and sodium, both being gradually less active as inducers (and in decreasing the transmembrane potential of the amoebae) (Fig 4) Concomitantly the increase in respiratory rate seen after reincubation in Pringsheim's solution was less marked The intensity of pinocytosis in KCl exhibited a slight increase during the same period of time.

Amoebae exposed to Tris—So 175 mM exhibited a stimulated rate of oxygen uptake. This was more prominent than the one noted upon reincubation in culture medium (Fig 2a) The increase at 20 minutes was about 100 per cent, half of which was still present at 40 min (Table I

TABLE I. Increase in rate of oxygen uptake in per cent of the resting respiratory rate observed in Pringsheim before incubation with solutions of Tris-SO and Na_4 EGTA at pH 7.0 and KCl at pH 5.7–5.8. The mean values are given ± standard error. Number in brackets denote the number of experiments with the diver technique. Control values in Pringsheim (pH 6.5) (line 1 and 6) refer to the experiments given in lines 2–5 and 7–10, respectively. To the right in the Table values for the intensity of pinocytosis during the first 20 min are given as the number of channels per amoeba.

Time (min)	15	20	25	30	40	Pinocytosis
1. Pringsheim (10)	91±32	55±17	24±16	10±14	−10±10	0
2. Tris-SO 175 mM (6)	143±34	107±21	87±20	77±22	56±15	3,1
3. Tris-SO 175 mM+	200	110	130	115	100	1 4
+EGTA 0.5 mM (2)	300	220	200	200	180	
4. Tris-SO_4 175 mM+						
+EGTA 10 mM (4)	67±26	69±26	52±73	38±20	24±13	0
5. Na_4 EGTA 10 mM (5)	126±37	73±3	47±15	40±15		0,1
6. Pringsheim (10)	50±22	28±14	26±15	9±12	5±15	0
7. KCl 60 mM (6)	102±19	74±16	47±12	31±4	0±8	5,6
8. KCl 175 mM (10)	65±16	56±19	28±9	22±7	6±8	3,4
9. KCl 60 mM+						
+$CaCl_2$ 5 mM (5)	127±50	29±14	13±18	9±18	−25±9	0
10. KCl 175 mM+						
+$CaCl_2$ 50 mM (6)	48±17	26±14	21±8	−4±12	−17±14	0

The inhibition of Tris pinocytosis by small amounts of calcium has recently been described in the previous paper (Josefsson 1968). In order to check whether or not the metabolic effects were parallel to the number of channels developed, this property of calcium was used in the experiments of Fig. 2b. The increase in oxygen uptake was significantly less in the presence of calcium. Magnesium, which compared to calcium is a weak pinocytosis inhibitor did not markedly alter the respiratory rate in the presence of 125 mM Tris.

Addition of calcium or magnesium chloride (10—30 mM) in the absence of Tris did not produce any substantial effect on the respiratory rate (Fig. 2b).

The measurements given in Fig. 2b are from the sensitive amoeba population, while those of Fig. 2a are from the less sensitive amoebae. Thus the differences observed in the increase of respiration in the two sets of experiments were not due to the different acids that were used to protonate the amine.

In two experiments with the cell in Tris-SO 175 mM + EGTA 0.5 mM a higher rate of oxygen uptake was observed than in the controls without the calcium chelating agent. The increase in oxygen uptake in this solution is shown in Table I and given from the 15th to the 40th min. The original respiratory rate was not reached until two hours after incubation. High concentrations of EGTA decreased both the number of channels and the oxygen uptake compared to the normal for Tris-SO as inducer.

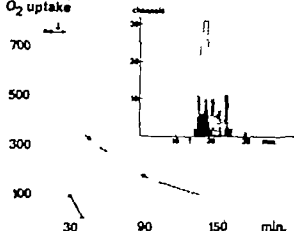


Fig. 3 Effect of calcium ions on the time course of the rate of oxygen uptake during pinocytosis. The bathing medium was changed at the arrow from the inducing solution (KCl 175 mM + $LaCl_3$ 20 mM) to this the same solution containing 15 mM $CaCl_2$ (solid line). Oxygen consumption indicated as in Fig. 2. Broken line indicates the result of an uninterrupted experiment with the inducing solution. Insert figure: number of channels observed per minute per amoeba in two 30 min pinocytosis cycles with the inducing solution (white bars). Hatched bars give the number of channels remaining after exchange at arrow to this inducer + 15 mM $CaCl_2$ (Solid bars = inducer + 30 mM $CaCl_2$). The mean of two experiments is given for the three conditions.

Potassium chloride is a good pinocytosis inducer at low concentrations and a maximum number of channels is observed with 30—40 mM. An increase of the potassium concentration to 175 mM reduces pinocytosis to about 50 per cent of the maximum. Measurements of oxygen uptake were made with 60 and 175 mM KCl. Of these concentrations the lowest was the best stimulator of respiration, increasing oxygen consumption by about 50 per cent above the control curve in Pringsheim solution 20 min after incubation (Fig. 2c). The addition of calcium to these potassium solutions abolished the number of channels and decreased the duration of the stimulated respiration (Table I bottom).

The alkali metal ions including calcium have the property of potentiating the sodium-induced pinocytosis at pH 5.8—7.0. Their effect is, however, limited to potassium concentrations above 50 mM and to certain divalent ion concentrations. When surpassing these concentrations the number of channels decreased (Josefsson 1968). A continuous progressive potentiation of pinocytosis was, however, observed with increasing concentrations of the rare earth metals lanthanum, cerium and thorium. These multivalent cations are weak inducers themselves although they potentiate pinocytosis elicited by any concentration of potassium. All the di- or tri-valent ions mentioned decrease or inhibit sodium induced pinocytosis. With solutions containing the chlorides of potassium (175 mM) and lanthanum (20 mM) at pH 5.5 we registered a very high rate of oxygen uptake (Fig. 2c). 20 min after incubation the increase was about 325 per cent, and 40 min still 165 per cent. The respiratory rate was generally normalized after 90 min. Lanthanum chloride alone gave no significant increase in oxygen uptake.

The presence of high concentrations of calcium was found to decrease the number of channels. In experiments in which pinocytosis was induced by KCl + $LaCl_3$, the cell was reincubated with the same inducer but with 15 mM $CaCl_2$ added. A decrease in the rate of oxygen uptake was observed 10—20 minutes after

introducing the calcium (Fig 3) In the insert of Fig 3 the time course of pinocytosis is given for the inducer and the effects of 15 mM and 30 mM $CaCl_2$ added during the 14th min. It should be noted that practically all the channels had disappeared at the time when the calcium-enriched medium was introduced.

The addition of lanthanum to media containing Tris or sodium did not increase the oxygen uptake accompanying pinocytosis, nor did lanthanum increase the number of channels developed.

Respiratory rate versus intensity of pinocytosis

The diver technique did not allow simultaneous measurements of respiration and pinocytosis. The latter had to be estimated on other cells than those used for respirometry. As an index of the intensity of pinocytosis the mean number of channels formed per amoeba during the first 20 minutes of incubation was used. The relation between the intensity of pinocytosis and the concentration of the inducer is given in the previous investigation (Josefsson 1968) From the dose response curves of Fig. 2 it is evident that dilution of the inducer during the procedure of refilling the diver would imply an increase of the potassium-pinocytosis at the concentrations used (60 and 175 mM) and a decrease of the Tris pinocytosis (125 mM) Dilution was estimated to be less than 25 per cent. The concentration of calcium ions originating from the culture solution (max 0.2 mM at 25 per cent dilution) should, according to the preceding report affect the pinocytosis intensity Slightly lower values of the pinocytosis intensity (compared to those given in Fig 4) should be expected in the Tris experiments while those with potassium should be unaffected or slightly increased.

The percentage increase in the rate of oxygen uptake present 20 minutes after incubation in eight different media was compared to the relative pinocytosis intensity during the first 20 min The results of such a comparison is shown in the

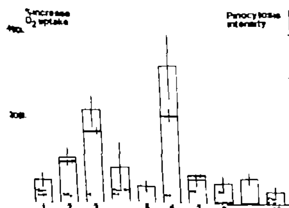


Fig. 4 Relative pinocytosis intensity measured from the first 20 minutes of the cycle. The respiratory rate taken at the twentieth minute is plotted in per cent of resting level. 1 Pringsheim (20/5) 2. Tris-SO 175 mM pH 7 0 (dec —66) 6/11) 3 Tris-Cl 125 mM pH 7.0 (febr —66 (8,14) 4 Tris-Cl 125 mM + $CaCl_2$ 1 mM pH 7 0 3 5 5 $CaCl_2$ 15 mM (3/3) 6 KCl 175 mM + $LaCl_3$ 20 mM 8 8) 7 KCl 175 mM (10 13 8 NaCl 175 mM + $LaCl_3$ 20 mM 3/5 9 $LaCl_3$ 20 mM (4/7) 10 Pringsheim (10/3) controls for the results given in column 7 Bars indicate S.E. of the means White columns indicate respiration. Hatched columns indicate pinocytosis intensity The number of measurements respiration/pinocytosis is given within brackets in the text

Fig 4 for four of the inducing salt solutions (columns 2 3 6 and 7) and for the inhibited pinocytosis (column 4) All cells responded to intensive pinocytosis by increasing their respiration. A constant relation between intensity of pinocytosis and increase of respiratory rate was not obtained. When calculated from the mean values at 20 min after incubation (Fig. 4) it varied between 5 to 20 per cent increase of the resting respiratory rate per unit channel developed, depending on the composition of the inducing solution. Inhibition of Tris-pinocytosis by calcium decreased respiration but the normal level was not attained (compare columns 3 and 4 in Fig. 4) Except for the two experiments with EGTA 0.5 mM added to Tris (see Table I) respiration was always increased under conditions suitable for intensive pinocytosis. As indicated in Fig. 4 (column 8) a mere increase of the ionic strength of the external medium does not affect respiration.

Discussion

Oxygen uptake in the amoeba proteus has previously been measured by Clark (1942) and by Brachet (1955) Clark found an average oxygen consumption of $1{\cdot}4 \times 10^{-3}$ μl/hr/cell (mean value from 10 measurements) Brachet, using amoebae cultured by Chalkley's technique, found an average oxygen uptake of 0.3×10 l/hr/cell (mean value from 15 measurements) Our value (2.5×10^{-3} μl/hr/cell) is in good agreement with Clark's. The difference between our figure and that of Brachet may be due to different culture techniques.

In electron micrographs of pinocytosis channels in *Amoeba proteus* Chapman-Andresen and Nilsson (1960) found mitochondria to be in direct contact with, or in the immediate vicinity of the channels rather than free in the cytoplasm of this ion. They suggest their function to be concerned with dehydration and/or tion of the fluid in the vacuoles. Irrespective of the tasks they perform, their n during pinocytosis propose a relation between pinocytosis and oxygen consumption Although a lot of metabolically important events secondary to pinocytosis deserves recognition, we ill limit our discussion to the direct effects of the two phases of pinocytosis pon resp ration.

When an amoeba was exposed to cations known to induce pinocytosis the oxygen uptake was increased. At the conditions studied using potentiated or inhibited pinocytosis, the respiratory increase aried according to the intensity of pinocytosis. A more than threefold increase of th normal respiratory rate was observed during intense pinocytosis. The ncrease in oxygen uptake was of about twice the magnitude as that reported to occur duri g phagocytosis (Sbarra and Karnovsky 1959)

Pinocytosis involves two phases th e adsorption of the cation to the surface of the amoeba and subsequently th in agination of the plasmalemma and acuolization of the channels.

For the second phase th dependence of pinocytosis on temperature and aerobic respiration has been described (de Terra and Rustad 1959) Our finding that both the development of channels and th timulated respiration approached zero 40

min after incubation is consistent with this view. The energy requirement of the second phase might therefore be the main cause of the observed increase in oxygen uptake.

It could be argued that the maintained stimulation of oxygen uptake during lanthanum potentiated potassium pinocytosis was caused by an effect of lanthanum on mitochondria similar to that reported for the rare earth metals by Horecker et al. (1939). The lack of effect of lanthanum applied alone or together with sodium, however, favours a relation between pinocytosis and respiratory rate.

Besides the energy requiring process of channel formation membrane permeability changes might increase the rate of oxygen consumption. An amoeba in culture medium exhibits a marked but shortlasting increase of respiratory rate when it is mechanically disturbed, exposed to pressure differences or flow of culture medium. These artifacts are reduced when the calcium concentration in the bathing medium is increased. The decrease in oxygen uptake when pinocytosis is inhibited by calcium and the high rate of oxygen consumption in solutions with low concentrations of EGTA (Table 1) might reflect changes secondary to the alterations in the ionic permeability of the membrane observed during the adsorptive phase of pinocytosis (Josefsson 1966).

The effect of calcium on the process of pinocytosis has been assumed to be at least twofold (Josefsson 1968): 1) Calcium associates with membrane receptors relevant for pinocytosis and prevents thereby the inducing action of a monovalent cation. 2) A channel forming action possibly exerted through activation of a contractile system responsible for the invagination of the membrane.

It is of interest to note that calcium ions seem to exert two opposite effects on the oxygen uptake as well as on the formation of channels. On one hand addition of calcium to the inducing solution decreased the respiratory stimulation. On the other the decrease in respiration observed in solutions containing high concentrations of a chelating agent and the subsequent recovery when calcium is added indicates that a minimal amount of calcium is required for stimulation of oxygen consumption, as well as for pinocytosis.

The generosity of Dr E. Giacobini, who gave training in the microdiver technique to one of us (O. J.) is gratefully acknowledged. We also wish to acknowledge the financial support from the Medical Faculty of the University of Lund and The Royal Physiographical Society of Lund.

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Role of Myogenic Propagation in Vascular Smooth Muscle Response to Vasomotor Nerve Stimulation

By

Borje Johansson and Bengt Ljung

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Abstract

Johansson, B. and B. Ljung *Role of myogenic p opagation in vascula mooth muscle ponse t asomot ne st mulation* Acta ph siol scand. 1968 73 501—510.

Innervated preparations of the spontaneously activ ascular smooth muscle of the rat portal ein were used for the pre ent study The preparation was so arranged to allow blockade of myogenic conduction between adjacent portions It was also possible to subject one of the portions selectivel to adrenergic eceptor blockade It ould be show hat myogenic propagation is necessary for coordination of the neu ogenic ontractile esponse as is the case with the synchronization of the spontaneous myogenic activity Moreover after the direct response to noradrenaline and adrenergic nerv stimulation had been blocked in one portion of the muscle selectively its activit as still enhanced during nerv stimulation by means of myogenic spread of excitation from the other portion her the innervation and adrenergic receptor function were intact I is oncluded tha myogenic onduction ontributes to the effectiveness of neurogenic activation by oordinating the ontractions and by recruiting non-innervated muscle ells to partic pate in the esponse The esult ar disc ssed in elation to the sparse innerv tion described in as lar smooth muscle

Vascular smooth m scle n many differe t essels show pontaneous h thmic contractions when studied i or isolated pr pa tion S h rh hmicit small precapillary essels, as seen b cal mic osc ips has bee termed asomotion It present n isolated preparations f o uch essel and al n trips f om some sections of the enous tree (for ref see g Wiedeman 1963 Bo h 196

It was show n a previous report that the spont neou h thmic ac y of the isolated rat po tal ein is due to myogenic utomatic y nd myogenic p ad of excitation J hansson and Ljung 196 b The nerv us elem ts f h el w ll are not essential f r generation or pr pagat on of he t f potentials which initia he contraction However timulat i he m nerves in rhythmical activ ascular smooth m scl medi h nh r au matic produces increase in th f equency and a pl t de f m ac ons (Johansson and Lj ng 196 It seems to be if teres t l da wh ther m ogenic

cellular propagation is of importance also for this neural response of the muscle. Since only a limited number of muscle cells are supplied with vasomotor nerve "endings" as indicated by the confinement of the adrenergic plexus to the outer most media in most vessels (Falck 1962, Ehinger Falck and Sporrong 1966) myogenic spread of excitation may markedly increase the effectiveness of the nervous control by recruitment of non-innervated effector cells.

The experiments presented below demonstrate that myogenic conduction is essential for effective coordination of the response of the portal vein to nerve stimulation and also that myogenic conduction is able to transfer the neurogenically induced excitation to muscle cells which are not directly affected by the adrenergic transmitter

Methods

The technique used in the present series of experiments constit tes combination of procedures utilized in previous studies on the nervous ontrol and on spread of excitation in the rhythmically acti ascular smooth muscle of the portal ein (Johansson and Ljung 1967 a, b) It as designed with the purpose of studying the concurrence of neurogenic and myogenic mechanisms in the neural excitatory response of this essel. I principle, this was achieved by ecording simultaneousl but separately the activity of t sections of the preparation, one with intact nerv suppl and another in which the adr ne gic receptors had been blocked. Muscle ontinuit was maintained between the innervated and the pharmacologically denervated" part and the m ogenic propagatin mechanisms were still functioning.

Af er preliminary experiments on nerv muscle preparations of the portal ein from both abbits and rats, the la species was hosen for the present study because the rat portal ein more readil showed regular and stable pattern of spontaneous acti ity and proved less sensiti to untoward terferenc from the experimental arrangements. These preliminary experiments indicated, however that the mechanisms described for the rat portal ein below are probabl alid for the rabbit essel as well.

The results p esented this report were obtained in 15 technically successful experiment n n -muscle preparations from the portal ein of male albino rats, weighing pproximatel g Sever l preparations had to be discarded because of damage to the essel or its nerv during dissection or mounting The animals were killed by blow on the neck. After bdomen had been opened, the portal ein was ligated and cut t equal distances f om ent ance of the splen ein The la ter essel had first been tied in lose proximity to its junction with he portal ein, whereby the midpoint of the portal ein section as supplied w h means of fixation which did not interfere with the tissue to be studied see further below The portal ein th it postganglionic nerv supply was then removed by careful dissection performed pr ncipall the same way as previously described f rabbits (Johansson and Ljung 1967 the only mportant difference being that the nerv fibres enter the essel mainly on he mesenteric side of he plexu ein the rat

The nerv -muscle preparation thus obtained was mounted in special thin-walled plastic organ bath (B in Fig 1) The hepatic end (h) of the ein was pulled through holes of suitable size burnt in t thin parallel rubber membranes (C) which formed part of the medial wall of the plastic hamber The l ga ure around the splenic branch was then tied t hoo on the outer side of this wall so th t the midpoint of the muscle preparation as fixed. The plastic bath was hen low red in mantled organ bath, whereby tw separate fluid compartments, A and B Fig 1 ere obtained, one for each half of the preparation. The tw free ends of the essel h and m were connected to force-displacement transducers (Grass FT03) and the isometric ension dev loped was recorded on Grass polygraph Each half of the ein was stretched to ts approximate length ax giving peak tension of 200 to 500 dyn. The mechanical activities of the sides could be recorded complete independent of each other with this arrangemen

To minimize the possibilities of diff sion bet een he t major fluid compartments, A and B the narrow central compartmen bet ee the rubber membranes, C, was continuously per fused w th prewarmed normal Krebs sol tion at slightly positi pressure By perfusing the central compartment, C th Krebs sol tion to which sucrose 300 mmoles l, had been added,

Fig. 1 Schematic drawing of the experimental arrangement. The hepatic (h) and mesenteric (m) ends of the vessel ... ork in tw fluid compartments A and B. The central chamber C, separated from A and B by thin rubber membranes, is continuously perfused to minimize exchange of solutions between A and B. Note the nerv bundle pulled inside the glass cannula and the two silver plates constituting ... fluid electrode.

the myogenic propagati ... mechanisms ... could be blocked selectively by the increase in osmolarity as earlier described (Johansson ... nd Ljung 196 b ...

The nerv bundle entering th mesenteric end of the preparation ... as pulled into ... glass cannula through ... hole in ... thin rubbe membrane covering its orific ... A bipolar fl id electrod, similar to that already described (Johansson and Lj ... g 1967 ... as obtained by placing one silver plate in the organ bath (B ... and ... nother inside ... he ... annula. The nerv fibres ... er stimulated by ... Grass S-5 stimulator giving square ... pulses of ... mpli ude ... nd duration tha ... ga ... maximal responses.

A modified Krebs sol tion ... as used ... ll experimen ... I ... had ... he follow ng ... omposition in mmole/l.

NaCl 122 KCl 4.73 $NaHCO_3$ 15.48 KH PO ... 1 19 MgCl ... 1 1 ... C ... ( ) ... F ... Glucose 11 ... and CaNa₂-versenate 0.026

It ... as continuous[ly] bubbled ... h ... ( ) ... O ... The tempe ... as kept consta ... 38 C

The following drugs ... sed ... l ... radr nalone ... Noreval ... A ... phenoxybenzamine HCl (Dibenyline Smith Kline ... d F ... nch ... and propr nolol HCl ... D ... are ... on expressed as ... eigh per ... olume ... l ed ... he base fo ... noradrenal ... e ... ... espec ... salt for the others

Results

The spontaneous acti ty of the ... portal ... s ll ... l ... l ... (... d periods of Fig 2. It is composed of ... es ... phas ... ... m ... h ... ter ... p ... d ... f rest during which the tension ... et ... ... table ... se ... l ... Th ... outp ... l ... d ... pol graph amplifier ... ecording the ac ... ... of he hepatic ... nd ... F ... ha ... b ... rted t bring the tw ... base-lines near ... thereby facilitating the ev ... f ... t on of ... h ... ... lt ... neous ness of the ... ecorded events. It is eviden ... from F g ... h t th ... ... m ... of th preparat on ... ontrac ... w th nearl ... comple ... ... ncl ... ning ha ... f ... tane ou ... ar ... ti ... ty

Upon ... t m lation of the ... sympathetic nerv ... ... re ... pons follow both pa ... of the ... vessel. This is demon ... ... rd ... F g ... where st mulat on ...

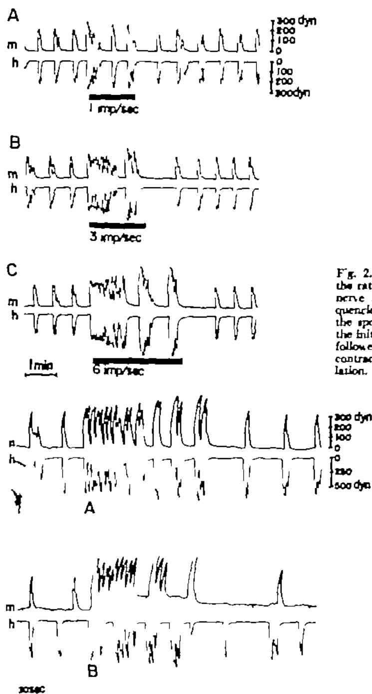


Fig. 2. Effects on the activity of the rat portal vein of sympathetic nerve stimulation at graded frequencies. Note the synchrony of the spontaneous contractions and the initial phase of tetanic activity followed by intermittent phasic contractions during nerve stimulation.

Fig. 3 Effects on the neurogenically induced activity of the rat portal vein of disrupting myogenic propagation between hepatic (h) and mesenteric (m) portion without interfering with neural connections.
A Sympathetic stimulation 6 imp/sec
B Sympathetic stimulation, 6 imp/sec while the central part of the vessel is exposed to 100 % increase in osmolarity. Note that the two sides contract independently of each other in B, but that nervous response is still obtained on both sides.

1.5 and 6 imp/sec were applied (4 msec, 15V). Initially a period of tetanic co[ntrac]tion is seen. During continued nerve stimulation the induced activity soon bec[omes] increasingly phasic in character and, eventually, intervals of complete rest reapp[ear]. The separate major contraction waves are now of significantly greater ampli[tu]d[e] than during the control period. The duration of the initial phase of continuous [ac]tivity is dependent on the stimulus impulse rate, as is apparent in the recordings of Fig. 2. In Fig. 3 A the response to nerve stimulation has been recorded at a higher paper speed. It is then clear that during the first part of the stimulation period the smaller individual contraction waves do not appear simultaneously on the two sides. They gradually become synchronous, however, being completely in phase by the time the intermittent type of activity returns.

To evaluate the importance of myogenic mechanisms in producing the coordinated response, the osmolarity of the external medium of the central portion of the [v]ein was increased. As earlier described, hypertonicity is capable of blocking th[e] myogenic cell to cell propagation without interfering with conduction in autonomic nerve fibres (Johansson and Ljung 1967 b). In Fig. 3 B sucrose, 300 mmoles/l, was added to the salt solution used in perfusing the narrow central compartment (C in Fig. 1). The spontaneous activity in Fig. 3 B then demonstrates a block of the myogenic conduction between the mesenteric and hepatic portions of th[e] muscle as

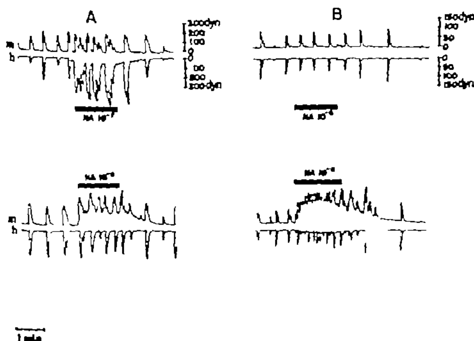


Fig. 4. The action of noradrenaline added to the ... selectively befor[e] the hepatic ... had been exposed to phenoxybenzamine 10

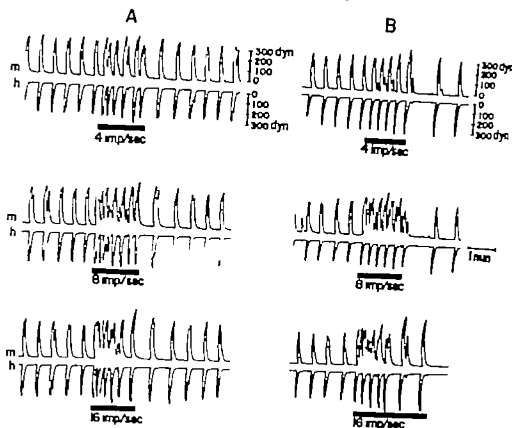


Fig. 5 The response of the rat portal vein to graded sympathetic nerve stimulation before (A) and after (B) the hepatic end had been exposed to phenoxybenzamine 10

... by the complete asynchrony of the phasic contractions. When the sympathetic nerves were stimulated an excitatory response was obtained in both portions which indicates intact function of the nerve elements. However the two parts of the vessel did not contract simultaneously as they did in Fig 3 A. It is thus evident that intact myogenic propagation is essential for the coordination of the vascular response to vasoconstrictor fibre stimulation, and that the neural connections alone are unable to execute this function.

In Fig. 4 the two portions of the preparation (h and m) were selectively exposed to noradrenaline before (panel A) and after (panel B) the adrenergic α receptors of the hepatic portion of the vein had been blocked by phenoxybenzamine 10 This concentration of the blocking agent is sufficient to eliminate the response to vasomotor fibre stimulation in this preparation (Johansson and Ljung unpublished observation). In Fig. 5 graded neural stimulations were performed before (panel A) and after (panel B) such an α-adrenergic blockade. A typical excitatory response was obtained from the portion which was directly exposed to noradrenaline in Fig. 4 A. An increase in amplitude and frequency of the contractions was obtained. However

the portion of the preparation supposed not to be in contact with the excitatory agent also displayed a marked increase in frequency contractions occurring eventually in association with the major waves of the exposed side. This response pattern may have two explanations. Either there was a leakage of noradrenaline between the two sides or there was a true propagation of the induced activity from the stimulated to the non-stimulated muscle portion. The validity of the latter explanation was shown in the following way. Noradrenaline was again added to the hepatic portion, after this had been exposed to phenoxybenzamine 10 No excitatory response then occurred on either of the two sides (Fig 4 B, upper recording). On the other hand, when noradrenaline was injected into the bath of the mesenteric portion, an excitatory response was seen, which indicates that the -receptors of this side had not been impaired by a possible leakage of phenoxybenzamine from the other compartment. Moreover there was now also a clear-cut increase in the frequency of contractions on the hepatic side. Since the α adrenergic blockade had been shown to be effective on this side the response cannot be ascribed to any leakage of noradrenaline but must be ascribed to myogenically propagated activity. Any possible role of adrenergic β-receptor stimulating effects of noradrenaline was excluded by adding propranolol 10 to the Krebs solution.

A good synchrony of the contraction waves of the hepatic and mesenteric portions of the portal vein was recorded in Fig 5 A, when the nerves to the vessel were stimulated. In an attempt to evaluate further the role of myogenic mechanisms in the coordination of the smooth muscle cells during the neural excitatory response nerve stimulations were repeated after the α-receptors had been blocked in the hepatic portion of the preparation by phenoxybenzamine 10 (Fig 5 B). The responses to stimulation in Fig. B ... essentially the same for the mesenteric portion, where the adrenergic α-receptor ... they were considerably reduced on the hepatic side. The ... which occurred in the blocked muscle portion must be ascribed to myogenic ... excitation from the portion where the adrenergic receptors were still intact ... from panel B in Fig 5 that the major contraction waves which ... well synchronized activity (Fig ... and 3 A ... to be propagated to the hepatic side. This may explain why the response ... completely to the blocked side than ... 16 imp/sec when activity is partly of the tetanic type. During the ... of the response to 16 imp/sec, in which intermittent phasic ... predominate, there ... effective conduction to the non-... muscle ...

Discussion

The effect of sympathetic nerve ... smooth muscle ... and rabbit portal vein were described ... report (Johansson ... 1967 ... isolated nerve-muscle preparation of the ... portal vein has been used in the present series of experiments. The ... spontaneous activity is more regular characterized by well defined ... contractions which are separated by

periods of quiescence. The response to nerve stimulation shows an initial period of tetanic activity the duration of which is dependent on the impulse frequency (Fig. 2) On prolonged stimulation the intermittent activity is resumed but the individual major contractions are now stronger and more long-lasting than in the control period. This response resembles, in all important respects, that obtained by moderate concentration (10 to 10 5) of noradrenaline in the bath (*cf* Johansson *et al.* 1967)

The purpose of the present investigation has been to elucidate the conditions of propagation in the portal vein under excitatory neural influence. This has been done by studying the synchrony of the contractile activity utilizing a modification of a method previously described (Johansson and Ljung 1967 b) The response-pattern observed in simultaneous recordings from the two sections of the vessel indicates a certain degree of desynchronization of activity during the initial part of the stimulation period (Fig. 2 and 3 A) It is likely that this reflects an influence of the vasomotor fibres on the automaticity of the vascular smooth muscle at several sites with in the preparation. This asynchrony is more pronounced with higher stimulation frequencies, probably due to an increase in the number of ectopic foci" There are, however even in the initial phase of the response, a number of major contraction waves, which occur simultaneously in the two muscle portions. The tendency to coordinated activity increases with time and, by some unknown mechanism, the muscle regains effective synchronization despite the continual influence of the transmitter An absolute prerequisite for this synchronized activity is, however that the mechanisms for myogenic spread of excitation remain intact, as is made evident by Fig. 3 B compared to 3 A. Both portions of the preparation still respond to nerv ulation when the central segment is being exposed to hyperosmotic solution. interrupts myogenic propagation. Coordinated contractions are absent ughout the timulation period as well as in the control state of spontaneous ity (Fig 3 B)

In addition to this ole of the myogenic mechanisms, for coordination of muscle activity under the drenergic influence, the present study has revealed an ability of the myogenic propagation to transfer the neurogenic excitation to muscle cells which are not directly affected by th transmitter This latter aspect is demonstrated by the experiments in which α-adrenergic blockade was selectively applied to one part of the muscle (Fig 4 and 5 Stimulation of the mesenteric part of the muscle by noradrenaline was thus associated with a clear-cut increase in activity also in the hepatic section whose direct response to noradrenaline had first been abolished by phenoxybenzamine (Fig. 4) A similar indirect activation of the α-blocked hepatic portion was obtained upon excitation of the mesenteric end by vasomotor fibre stimulation (Fig. 5) These results must mean that muscle cells on the hepatic side were recruited to participate in the adrenergic response by myogenic spread to excitation from the mesenteric portion This mechanism appears to be more effective the better the synchronization in the primarily activated muscle portion. The quantitative importance of the myogenic recruitment cannot be evaluated simply by comparing the size of the responses of the mesenteric and the hepatic side n Fig

5 B. The reason is that myogenic mechanisms are likely to contribute also to the response of the "innervated" muscle portion itself since only a fraction of the muscle cells are in contact with nerve endings in the portal vein (Fuxe, personal communication).

The conclusions, based on the present results, imply in summary that myogenic propagation is important both for the coordination of the vascular smooth muscle response to stimulation of its sympathetic nerve supply and for the recruitment of muscle cells which are not directly affected by the adrenergic transmitter. Diffusion of the transmitter is another possible mechanism by which muscle cells without direct nerve supply might become excited during sympathetic stimulation but it is probably of little importance at physiological impulse rates due to the effectiveness of the active transmitter re-uptake into the axons (Folkow, Häggendal and Lisander 1967). "Over-flow" of transmitter was found at supraphysiological frequencies (above 10 imp/sec). Signs of noradrenaline diffusion to deeper layers of the media in dog dorsal pedal artery were found histochemically by Gerová, Gero and Doležel (1967) at sympathetic stimulation with 15 imp/sec and this was considered of functional significance.

The fact that neurogenic and myogenic mechanisms are interlinked, in the way demonstrated for the portal vein by the present series of experiments, is likely to bear on vasomotor control in general. The haemodynamically important precapillary resistance vessels, for instance, are characterized by a myogenic "tone" (e.g. Folkow 1964 a, b) which can be ascribed to the presence of smooth muscle of the propagating type. Only the very outer layers of the media of these vessels appear to be directly innervated by adrenergic fibres (Falck 1962, Folkow, Öberg and Rubinstein 1964, Fuxe and Sedvall 1964). This arrangement implies that the muscle cells of the inner layers are able to maintain an autonomous myogenic vascular tone and to effectuate such vascular adjustments as myogenic reactions to changes in passive stretch and dilator responses to local metabolites. They can, on the other hand, by myogenic spread of excitation from the outer innervated layers enhance the constrictor response to sympathetic activity. Intensification of the vasoconstrictor fibre discharge may therefore imply a centralization of vascular control at the expense of local mechanism.

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A Method to Determine Osmotically Effective Albumin and Gammaglobulin Concentrations in Tissue Fluids, Its Application to the Uvea and a Note on the Effects of Capillary "Leaks" on Tissue Fluid Dynamics

B

Anders Bill

Received 29 December 1967

Abstract

Bill, A. *A method to determine osmotically effective albumin and gammaglobulin concentrations in tissue fluids, its application to the uvea and a note on the effects of capillary "leaks" on tissue fluid dynamics.* Acta physiol. scand. 1968. 73 511—522.

Choroids and ciliary processes from rabbits were incubated with labelled myoglobin, albumin and gammaglobulin and the equilibrium spaces were determined. Using experimental data for the ... steady state myoglobin space ... tissue an approximate ... for the ... equilibrium space could be calculated. The ... albumin and gammaglobulin equilibrium spaces were calculated using the data ... equilibrium myoglobin spaces and the differences in spaces determined ... for the effective albumin and gammaglobulin concentrations in the tissues ... by dividing the ... spaces by the calculated ... equilibrium ... The choroid the effective albumin and gammaglobulin concentrations ... 48 per cent of ... plasma ... the ciliary processes the corresponding ... 4 and 6 per ...

Model experiments showed ... membranes ... 500 Å pores ... colloid osmotic pressure of rabbit plasma ... albumin and ... gammaglobulin ... cm H_2O.

The results indicate ... the choroid and the ciliary processes ... net outflow of water in the semipermeable ... the blood ... hydrostatic pressure difference of more than ... mm Hg ... pores permit ... is required to ... net ... fluid ...

According to Starling's hypothesis the ... flow of water out of a capillary is influenced by the hydrostatic pressure difference ... the capillary wall and the difference in colloid osmotic pressure between plasma and the gelatinous tissue fluid. Methods for determination of the hydrostatic pressure in easily exposed capillaries (Intaglietta and Zweifach 1966) and the colloid osmotic pressure of plasma have been described and information about the hydrostatic pressure in the tissues is accumulating (Guyton 1963, Guyton et al. 1966). It is known that large amounts

plasma protein are located in the extravascular fluids but little is known about the protein concentrations in the different tissue fluids (see review by Landis and Pappenheimer 1963)

In rabbit eyes the hydrostatic tissue pressure must be about the same as the intraocular pressure, that is about 20 mm Hg This raises the question as to how there can be a net movement of fluid out of the uveal capillaries. Evidently if Starling's hypothesis applies there must be a very high average hydrostatic capillary pressure if there is not a high protein concentration and thus a high colloid osmotic pressure in the tissue fluid.

In a previous study (Bill 1968) it was found that the steady state plasma equivalent albumin and gammaglobulin spaces in the choroid and the ciliary processes are compartively large and the dynamics of radioactive myoglobin in the tissue fluid was such as to indicate that in steady state the plasma equivalent steady state extra ascular myoglobin space represented more than 80 per cent of the equilibrium space a space hich cannot be determined in vivo The plasma equi a lent steady state albumin space in a tissue sample was calculated as the amount of radioacti e albumin in the sample at steady state divided by the amount of radioactive albumin per *µ*l plasma. An analysis of the data for the accumulation of the radioacti e substance in the tissue made it possible to calculate intra ascular and extravascular portions of the spaces.

The present study attempted to determine the osmotically effecti e concentrations of albumin and gammaglobulin in the tissue fluids of the choroid and the ciliary processes. The calculation is based on previous data from *in vivo* experiments (Bill 1968) and on data from *in vitr* experiments which are reported here Some model xperiments designed to elucidate the effects of capillary leaks are also reported.

Methods

I t perim t

Enucleated eyes from llano albus, body weight 2—3 kg were dissected as has been described in previous papers Bill 1964 1968) The preparations used re the ciliary processes, the rest of the anterior uv a nd the choroid The preparations were incubated and agitated at 2 C in an ncubation fluid tha contained [131]I-myoglobin and [131]I-albumin or I-gammaglobulin and [14] I albumin Af er 1 to 4 hrs the tissue samples were collected from the incubation fluid, washed w h inactiv ncubation fluid for one sec and then blotted on filter paper and put int pre-weighed pol ethylene ubes and weighed I other experiments in an attempt t avoid losses of high molecula weight tissue constituents the preparations ere enclosed between Millipore membranes pore size 0 45 µ (Millipore Filter Corp W tertown, Mass U.S.A.) The membr nes are permeable to proteins but not to l aluronic and (Laurent 1964 Vaseline was sed fill the space between the membranes that was not occupied by the prepara ions After 24 hrs ncubation one of the filters was removed and the preparation was washed w th inactiv incubation fluid for one sec blotted w th filter paper and collected from the fil er I some of the experiments with albumin nd gammaglobulin the p parations were not washed afte the ncubation, only blotted

The amount of labelled material cpm a tissue sample was divided by the concentration of the substanc in cpm/µl he incuba ion fluid. The ratio, in µl per preparation, represents the space of the substance the preparation. The spaces are apparent since they do not represent welldefined lumes Limits show are ± one standard error of the mean. *Sol tions.* [131]I-albumin was obtained from AB Atomenergi, Studsvik. Horse myoglobin, human and rabbit IgG-gammaglobulin were labelled w th [131]I by Dr K Hellung (Bill and Hellung

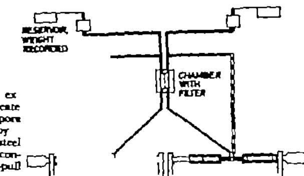


Fig. 1 The set-up of the model ex periment. T chambers communicate th each other through millipore membrane stabilised on each side by 0.05 mm thick perforated stainless steel membrane. Both the chambers are connected to reservoirs and push-pull coupled syringes.

1963) The incubation fluid in most experiments was rabbit plasma diluted with isotonic NaCl and isotonic NaI in the ratios 1 0.9 0.1 The NaI was added to educe any activ accumulation of radioactiv iodine in the preparations. I experiments with myoglobin 10 mg/ml arrer myoglobin was added. The pH was adjuster to 7.4 Gel filtration with Sephadex G 25 as used before each experiment to remove any radioactiv iodine not bound to the protein and at the end of some experiments samples of the incubation fluid were analyzed by the same procedure to check that significant amounts of radioactiv iodine had not separated from the protein. In some experiments the labelled gammaglobulin and albumin used were "screened" by injection into the animal that was to deliver the plasma for the incubation. The injection as made 30 min befor the bleeding. The plasma was used undiluted.

Gammaspectrometry was used to analyze the samples for ^{125}I and ^{131}I The counting error was less than 5 per cent for each isotope for all samples.

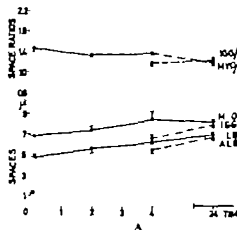
Model periment

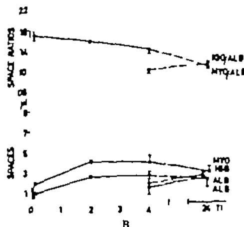
T obtain an estimat of the effects of differences in protein concentration on the difference in colloid osmotic pressure over porous membrane with 500 Å pores some model experiments ere performed. Fig. 1 show the se p of these experiments. A millipore membrane pore size 470—530 Å according to the manufact rer was placed between tw hambers The membrane was stabilized on both sides by hi perforated sheets of stainless steel. The contents of each camber could be agitated using tw push-pull coupled syringes Both sides of the chambers were connected to reservoir containing the same fluids as the syringes The weight of each reservoir was recorded continuously (Barány 1966 and he reservoirs could be adjusted at different heights below he hambe contain g the millipore membrane Net movement of fluid from one system the other as reflec ed as ain of weight of one reservoir and corresponding loss of igh from he other A he tart of he experiments the fluid on one side of he membrane as f esh abbit plasma ha on the other side was 0.9 per ent NaCl. Both reservoirs wer 3 m below the millipore f lter There was some flow from the NaCl-side to the plasma side The heigh of the NaCl reservoir as hen changed and the resulting flow ates determined Some experimen also performed with 3 per cent albumin or 3 per cen huma gammaglobulin 0.9 pe en NaCl of one side and 0.9 per cent NaCl on the other The experiments er performed at room temperat re of 23 C.

Results

I itro xperiment

The ch oid In the experiments with albumin nd gammaglobulin the r io of the gammaglobulin and albumi spaces was lculated for each preparati The myoglobin/albumin pace ratio was calculated similarly Fig 2A show h w th spaces and pace ratios hanged w th the time of incubation. T ble I shows the erage 4 hr paces. The mean weight of th preparations was 1_6 ± 0.6 mg The





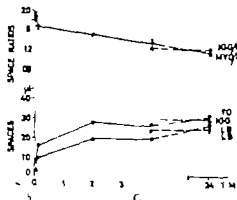


Fig. 2. Choroids, A, ciliary processes, B and the rest of the anterior uv preparations C ere incubated for different times in solutions containing labelled myoglobin and labelled albumin or labelled IgG-gammaglobulin and albumin. The apparent protei spaces in the preparations are shown. The ratio myoglobin space/albumin space or IgG space/albumin space as calculated for each preparation The space ratios are shown. Points represent arithmetic means of t least 5 preparations. The ertical bars show the standard error of the means.

mean value for the 24 hr experiments for the difference myoglobin space—albumin space was 1 2 μl. The mean difference gammaglobulin space—albumin space was 1 1 μl.

The ciliary processes Fig 2B shows the gammaglobulin/albumin space ratio and the myoglobin/albumin space ratio and the protein spaces calculated as in the previous preparation. The 24 hr incubation of ciliary processes ga e myoglobin spaces that were rather different from th 4 hr experiments. This as due to technical difficulties it proved impossible to collect all of the ciliary processes from the filter after the incubation. As a consequence the a erage weight of the preparations collected was 5.40 ± 0 73 mg as compared with 7 60 ± 0.83 mg in the 4hr experiments. In the calculations of the a erage differences between the spaces the loss of material was taken into account by multiplying the 24 hr differences observed by 7 6/5 4 The experiments with albumin and gammaglobulin were also affected by loss of materal for which a similar correction was made The mea difference myoglobin space—albumin space in the 24 hr experiments was 0.41 μl and the difference gammaglobulin space—albumin space was 0 18 μl. The spaces shown in Table I ha e been corrected for the loss of material

TABLE I The In ᴠitro equilibrium spaces, the In ᴠivo steady stat spaces and the calculated in equilibrium spaces for myoglobin, albumin nd gammaglobulin. The effective concentrations for albumin and gammaglobulin in the tissue fluids are expressed as percentage of the plasma concentrations. For the I ᴠitro albumin spaces tw figures are given, the first was determined in experiments with myoglobin, the second in experiments with IgG-gammaglobulin.

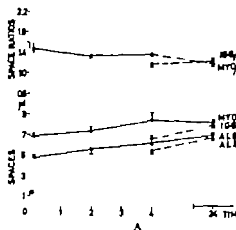
		In ᴠitro eq sp. µl	In ᴠi st.st.sp. µl	I ᴠiᴠ eq.sp. µl	Eff. prot.conc. percent of that in plasma
Choroid.	Myoglobin	8.1	5.9	6.5	90
	Albumin	6 8	3.6	5.3	68
	Albumin	6.9	—	—	—
	IgG	7.9	3.1	6.4	48
Cil proc.	Myoglobin	4.2	2.1	2.3	90
	Albumin	3.8	1 4	1.9	74
	Albumin	4 8	—	—	—
	IgG	5.0	1 4	2.1	67
Rest of anterior uvea					
	Myoglobin	30 2	4.9		
	Albumin	25.8	2.6		
	Albumin	24 7	—		
	IgG	29.2	3.1		

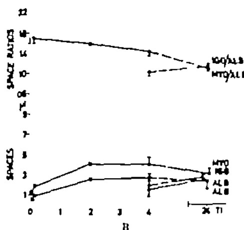
The est f the anterior vea Similar data as the above f r this preparation are shown i Fig. °C and Table I. The mean weight of the preparation was 50.9 ± 2.8 mg.

Controls These experiments were performed to in estigate if the unexpectedly large gammaglobulin space could be explained by the washing of the preparations, the use of human serum gammaglobulin or through denaturation of gammaglobulin. However the results of 3 experiments with screened' albumin and gammaglobulin were not different from the others The gammaglobulin space was somewhat larger than the albumin space. This was the ame also w th th preparations which were only blotted after the incubation. The results of the experiments w th rabbit gamma globulin also were not significantly different from those with human gammaglobulin.

Model perim nts

Ten experiments were performed Th accuracy of the flow determinations was such as to give the true rate of flow ±0 µl/min. I all experiments less than 1 cm H O hydrostatic overpressu in the hamber w th protein was enough to top ny net flow from the saline side towards the protein sid The pparent colloid osmotic pressure of the three protein sol tions was thus less th n 1 cm H O F g. 3 shows typical results.





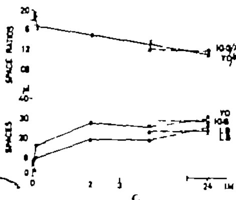


Fig. 2 Choroids, A, ciliary processes, B and the rest of the anterior ea preparations C were incubated for different times in solutions containing labelled myoglobin and labelled albumin or labelled IgG-gammaglobulin and albumin. The apparent protein spaces in the preparations are shown. The ratio myoglobin space/albumin space or IgG space/albumin space as calculated for each preparation. The space ratios are shown. Points represent arithmetic means of at least 5 preparations. The ertical bars show the standard error of the means.

mean value for the 24 hr experiments for the difference myoglobin space—albumin space was 1 2 μl. The mean difference gammaglobulin space—albumin space was 1 1 μl.

The ciliary proc sses Fig 2B shows the gammaglobulin/albumin space ratio and the myoglobin/albumin space ratio and the protein spaces calculated as in the previous preparation The 24 hr incubation of ciliary processes gave myoglobin spaces that were rather different from the 4 hr experiments. This was due to technical difficulties it proved impossible to collect all of the ciliary processes from the filter after the incubation. As a consequence the a erage weight of the preparations collected was 5 40 ± 0 73 mg as compared with 7 60 ± 0 83 mg in the 4hr experiments. In the calculations of the verage differences between the spaces the loss of material was taken into account by multiplying the 24 hr differences observed by 7 6/5 4 The experiments with albumin and gammaglobulin were also affected by loss of materal for which a similar correction was made The mean difference myoglobin space—albumin space in the 24 hr experiments was 0 41 μl and the difference gammaglobulin space—albumin space was 0 18 μl. The spaces shown in Table I ha been corrected for the loss of material.

follow it is assumed that all the radioactive protein recovered in the preparations was present in the tissue fluid and that only sterical effects influenced the distributions. This makes the data calculated for gammaglobulin less reliable than those for albumin. The error to be expected for gammaglobulin will be a moderate underestimation of effective concentrations.

The effective protein concentration in the interstitial fluid

In 1962 Creese, D'Silva and Shaw reported a technique to obtain samples of tissue fluid from muscles in anesthetized animals. The tip of a small cannula was blindly introduced into the muscle. In one of ten trials a small amount of fluid, usually 0.1—0.2 µl, entered the capillary. The fluid was assumed to be tissue fluid. The average albumin concentration in the fluid collected was 42 per cent of that in serum. The globulin concentration was very variable. In many other studies, lymph collected from cannulated lymph vessels has been assumed to represent interstitial fluid. This, of course, is an approximation since the interstitial fluid changes in composition on its way into and through the lymph vessels.

When colloid osmotic pressures and tissues fluid dynamics are discussed it is the osmotically effective concentrations of the proteins that are of primary interest. Due to steric exclusion effects of the macromolecules in the ground substance, proteins do not have access to the whole volume of the extracellular water (Ogston and Laurent 1963, Laurent 1964). As a consequence it can be expected that the osmotically effective concentrations of proteins in the regions to which they have access will be higher than expected on a strict protein weight to tissue fluid volume basis.

In the following an attempt is made to determine approximate values for the effective protein concentration in the tissue fluids.

The reasoning is as follows. If a tissue sample could be incubated for a long time in plasma without changes in tissue fluid volume equilibrium in protein concentrations would be attained between the tissue fluid and plasma. The effective albumin concentration EC in all parts of the tissue fluid would be about the same as the concentration in plasma. The equilibrium extravascular space S_{eq} could then be defined as the total amount of albumin in the tissue fluid A_{eq} divided by the concentration of albumin in plasma C_{Pl}

$$S_{eq} = A_{eq}/C_{Pl} \qquad (1)$$

In vo, albumin is drained from the tissue and as a consequence the steady state effective albumin concentration the tissue tends to be lower than it would be if it were in equilibrium with the same plasma. The steady state albumin space of albumin, S_{ss} is defined as the total amount of albumin in the tissue fluid divided by the plasma concentrations, C_{Pl}.

$$S_{ss} = A_{ss}/C_{Pl} \qquad (2)$$

Since also in a steady state albumin is distributed in the volume S the effective concentration of albumin in the tissue fluid is A_{ss}/S_{eq}. If the effective albumin concentration in the tissue fluid is EC_{alb} per cent of that in plasma it follows from (1) and ( ) that

$$EC_{alb} = 100 \quad S_{alb}/S_{plA} \quad (3)$$

Effective concentrations for gammaglobulin and myoglobin may be determined analogously

In the experiments reported here absolute values for the protein contents were not determined but the experiments gav values for the *in vitro* equilibrium spaces for albumin myoglobin and gammaglobulin.

The weights of the *in vitro* preparations were different from the previous *in vivo* preparations. The reason for the discrepancy is not quite clear It is possible that the *in vitro* preparations contained some vitreous and aqueous humor with very little activity and/or that the *in vitro* preparations lost weight due to evaporation. The difference in weights makes it impossible to compare spaces per gram tissue the different spaces in the same preparation seem to be a more reasonable ground for comparisons.

Effective albumin and gammaglobulin concentrations in the choroid and the ciliary processes may then be calculated as follows In the choroid the *in vitro* equilibrium space for myoglobin was 1 2 µl larger than the albumin space and the gammaglobulin space was 1 1 µl larger than the albumin space. It is assumed that the *in vivo* steady state extravascular myoglobin space is about 90 per cent of the *in vivo* equilibrium space, since it has been shown that the space is most probably more than 80 per cent sat rated (Bill 1967) Using the value of 270 µl/g for the *in vivo* steady state myoglobin space and the mean weight of the *in vivo* preparation 21.8 mg the *in vivo* equilibrium space becomes 6.5 µl. Assume now that also the *in vitro* myoglobin space is 1.2 µl larger than the equilibrium albumin space then space is 5.3 µl. The gammaglobulin space was 1 1 µl larger than the albumin i.e. 6.4 µl These assumptions seem justified since, at least in sol tions of hyaluronic acid the olumes from which proteins seem to be excluded are very little red by moderate variations in the water content of the sol tion (Laurent 196) Using the al es for the *in vivo* st ady tate spaces of albumin and gammaglobulin the effecti e concentrations can then be calculated according to equation 3. This procedure can be also used for the ciliary processes.

In the rest of th anterior ea preparations which contained iris tissue and small amounts of the ciliary body the *in vivo* steady state spaces were ery much lower than the *in vitro* pace T ble I) although the weights of the preparations were not very different 45 6 ± 1 mg *in vivo* 50 9 ± 2.8 mg *in vitro* This show that it is impossible to calculate pproximate effecti e protein concentrations in this complex preparation. The reason for the low *in vivo* spaces is no doubt that ery little protein passes out of the bl d essels of th iris (Bill 1968) and whatever comes out most probably passes rapidl into the anterior chamber which seems to communicate more or less freely with the fluid in the iris due to the incompleteness of the endothelium covering the ant rior surface of the iris. Histologically a marked difference in protein concentration between the ciliary processes and the iris stroma has been demonstrated by Ehinger Dyster Aas and Krakau 1965

The role of large pore in the apillary wall

The effective protein concentrations calculated above are averages for the tissue fact there may be local differences in protein concentrations and the effect of protein.

If the major part of the protein leakage occurs through temporarily open cleft between the endothelial cells as suggested by the results of Landis (1964) it can be presumed that if one capillary and its venule are discussed, the wall of the blood essel is in principle a nearly ideal semi-permeable membrane to solutions of albumin and gammaglobulin with a few imperfections. These imperfections are most probably identical with the 40—700 A "leaks" of Crotte (1956). In the ideally semi-permeable part of the capillary including the enule the net movement of fluid is determined by the hydrostatic pressure and the effecti e colloid osmotic pressures that ha e been discussed above. At the spots however where also proteins can pass through the wall the situation is more complicated. This is illustrated by the results of the model experiments. They showed that the apparent colloid osmotic pressure of rabbit plasma over a membrane with 500 Å pores is less than 1 cm H O. The colloid osmotic pressure of rabbit plasma is about 21 mmHg (for references see Yoffey and Courtice) and those of 3 % albumin and 3 % gammaglobulin solutions are about 10 and 3 mmHg respectively (Landis and Pappenheimer 1963). The low apparent colloid osmotic pressures observed were no doubt due to the fact that osmotic effects over a membrane permeable to both the solvent and the solute is less than expected from an t Hoffs law (Sta ermann 1951). Durbin (1961) has studied th relationship between the reflection coefficient and the ratio molecular diameter of solute/ diameter of pore fa some artificial membranes with pores ranging from 23 to 82 A. With ratios of 0.1—0.2, which would be the relevant figures for albumin and gammaglobulin in 500 A leaks the reflection coefficients expected from an extrapolation of Durbin results would be 0 1—0 3 that is the osmotic effects would be 10 to 30 per cent of those exerted by a nonpenetrating substance w th the same molar concentration. The present results indicate an even lower figure of about 0.0 —0.1 for the reflection coefficients. Thus in the blood essels the osmotic effects of the plasma proteins on the flow through the large pores, or leaks can be expected to be lower than on that through small pores. This means that "leaks" in the enous part of the capill ry and maybe even in the enules may h a net movement of water and solutes out of the essel while nearby small pores ha e a net movement of water in the other direction. This gi es local irculation of fluid out of and back into the enous part of the capillary nd the enule in addition to the flow from the arterial part of the capillary towards the enous part Fig 4. In capillaries w th precapillary phincters when the sphincter is closed there will be net inflow into the vessel through all small pores and outflow onl through the large ores.

It can be expected that the opening of large pore in the enous part of the capillary or in a enule will t nd to gi net flow from this part of the capillary or enule nto the l mph essels or other paths draining tissue fl id. With this fl will be carried also high molecular weight substances that ha tended to

late outside the venous part of the capillary during the period with only small pores opened. One may speculate that the accumulation outside the vessels of such substances may be the factor that stimulates the opening of the large apertures and the subsequent washing away of the substances in question.

The above hypothesis may help to explain why in some tissues plasma proteins in the interstitial fluid seem to return into the blood vessels within the tissues only to a very small extent if at all (Review by Yoffey and Courtice 1956, Paldino and Hyman 1966, Bill 1968). It seems quite possible that in such tissues most of the pores that permit the passage of protein have a net outward flow of slightly modified plasma that very strongly counteracts inward diffusion of protein located outside the vessels. It should be pointed out that unidirectional pinocytosis out of the lumen of the blood vessels is an alternative possibility that would give a local circulation of low molecular weight substances of the type outlined above and no return of proteins through the capillary wall.

Net movement of fluid through the uveal vessels

In the ciliary processes the osmotically effective albumin and gammaglobulin concentrations in the tissue fluid seem to be about 70 per cent of those in plasma (Table I). The total colloidosmotic pressure of rabbit plasma is about 21 mm Hg (for references see Yoffey and Courtice 1956). If the osmotic pressure due to the plasma colloids were linearily related to the concentration of the colloids the colloidosmotic pressure of the tissue fluid would be 70 per cent of 21 mm Hg, that is about 15 mm Hg. In fact there is some nonlinearity (see Landis and Pappenheimer 1963). When ...s is allowed for the figure for the colloidosmotic pressure exerted by the extra- ... plasma proteins becomes about 12 mm Hg. The ground substance macro- ... can be presumed to contribute somewhat to the colloidosmotic pressure. ...alue of 14 mm Hg is a reasonable figure for the total colloidosmotic pressure of the fluid outside the capillaries.

It may be concluded then that a transmural hydrostatic pressure difference of about 7 mm Hg is enough to give a net flow of water and low molecular solutes out of a blood vessel in its semipermeable parts and at 500 Å leaks less than 1 mm Hg is required. *In the choroid the situation seems to be similar to that in the ciliary* processes but in the iris capillaries the transmural hydrostatic pressure difference and the colloid osmotic pressure of the tissue fluid are probably not high enough to balance the high colloid osmotic pressure of plasma. Here, however, as Kinsey and Reddy (1964) have pointed out the tendency to water movement out of the capillaries due to the hypertonicity of the anterior chamber aqueous humour may very well overcome a tendency to net water reabsorption.

The method to determine effective protein ... n ... nl ation

In tissues with a low rate of myoglobin turnover or a high rate of myoglobin drainage with lymph a smaller molecule than myoglobin may have to be used to determine an *in vitro* steady state space that is close to the *in vivo* equilibrium space of the

molecule. This information is needed as a link between the *in vivo* and spaces. The smaller the reference molecule the greater will the risk be tha error in the calculations of the *in vivo* equilibrium albumin and gammagl spaces. In cell-rich tissues penetration of plasma proteins into damaged cell make *in vitro* space determinations useless, but even then approximate albumin gammaglobulin concentrations can be determined from appropriately chosen *in* spaces. Thus for the tissues discussed here the extravascular *in vivo* steady state albumin space divided by the extravascular *in vivo* equilibrium myoglobin spac gives an effective albumin concentration of 55 per cent of that in plasma for the choroid and 60 per cent of that in plasma for the ciliary processes. These values are lower limits, however since as has been pointed out albumin and gammaglobulin tend to be excluded from parts of the myoglobin space.

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Acta physiol. scand. 1968. 73. 523—526

From the Department of Physiology Karolinska Institutet, Stockholm, Sweden

Maintenance of Noradrenaline Synthesis in Rats after Reserpine Treatment

By

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Abstract

Wennmalm Å. *Maintenance of noradrenaline synthesis in rats after reserpine treatment.* Acta physiol. scand. 1968. 73 523—526.

Studies of the excretion i the urine of noradrenaline (NA) and 3-methoxy-4-hydro ymandelic acid (VMA) during 5 day treatment with 0.05—0.1 mg/kg of reserpine in rat with demed l lated adrenal glands are reported. The excretion of NA was moder tely reduced bu t VMA excretion as not significantly influenced by this treatment lthough the heart was almost completely depleted of NA within 48 hours. These results indic te that synthesis of NA may continue in spite of reserpine treatment causing tissue depletion of the amine suggesting he normal storage is not prerequisite for resynthesis.

The question whether reserpine influences NA-synthesis is at present nder debate Kirshner Rorie and Kamin (1963) found that reserpine in a dose of 2.5 mg/animal in the cat inhibited the transport of dopamine into ad enal med llary gra ules and thus the subsequent conversion to NA. Similar results w re r ported by Stja ne and Lishajko (1966) from experiments with isolated bov ne splenic nerv granules and by Werner and Rutledge (1966) and Rutledge and Wei ner (1967) in the base of observations made on adrenal medullary slices and isolated perf sed ... heart. Stjarne, Roth and Lishajko (1967) also reported that the inhibit on of NA ... was dependent of the local reserpine/dopamine rate r the medi m surrounding ... nerv granules, indicating that the reserpine-ind ced nh bition of NA ... competit e in nat re.

In the present study the rinary excretion of NA and VMA ... treatment w th reserpine in low doses dur ng 5 ... ive da ... of the heart was used as an index of the tissue depletio of NA and ...

Material and methods

Male rats of he Sprague-Dawley ... an, weighing 2.0 120 g were ... The s used for studying the influence of reserpine on the NA ... divided w groups, ach at receiving daily dose 0 ... (Serpasil® Ciba ir peritoneall From ach group, wo ...

head after 24 48 and 72 hrs. The hearts were immediately removed and homogenized by an Ultra Turrax pparatus and extracted with 10 % trichloracetic acid. The extracts ere purified by alumina and assayed for NA according t Euler and Lishajko (1961)

Rats used for studyi g reserpine influence on urinary excretion of NA and VMA were bilaterally adrenodemedullated according to Biscardi Carpi and Orsinger (1964) Urine collection was initiated 5—12 day after the surgery

The animals were placed i individual cages mounted so that urine collection was possible without contamination with food or feces. Urine was collected in 5 ml N sulphuric acid.

The rats used for rine collection were divided in 3 groups, each rat receiving daily dose (0.05 or 0.10 mg/kg) intraperitoneally of reserpine or drug solvent in equivalent olume for 5 days. The collected 24 hrs rine was centrif ged, purified on alumina and assayed for NA. The effluents from the alumina columns were assayed for VMA according to Pisano, Crout and Abraham (1962) The overall recovery for NA ranged between 70 and 80 per cent and for VMA between 95 and 100 per cent.

Results

In 24 hrs, the NA in the heart decreased to about 50 per cent and in 48 hrs to about 7 per cent of the normal NA values during treatment with reserpine at a dose of 0.05 mg/kg The higher dose 0 10 mg/kg decreased the heart levels to about 30 per cent in 24 hrs and to about 4 per cent in 48 hrs (Fig. 1) Reserpine treatment with either dose for 5 days had no significant effect on urinary VMA (Fig. 2) while the NA excretion was moderately reduced by 0 05 mg/kg and considerably diminished by 0 1 mg/kg

Discussion

The present results are in good agreement with earlier observations of the effect of reserpine on NA in the heart (Bertler Carlsson and Rosengren 1956 and thers) VMA, a metabolite of NA as shown by Armstrong and McMillan (1957) contes together with normetanephrine and 3 methoxy-4-hydroxy phenylglycol the ›⁴tati ely most important metabolite. Since the animals were demedullated the tribution of adrenaline to the VMA in urine was presumably greatly diminished.

Excretion of VMA in man after a single dose of reserpine has been measured by McDonald and Weise (1962) After a dose of 2.5 mg they found a rise n VMA excretion within 8 hrs. During the following 16 hrs the excretion of VMA was not significantly altered. It was concluded that the initial rise in VMA excretion was due to metabolism of the catecholamines released by reserpine. Excretion of NA in man

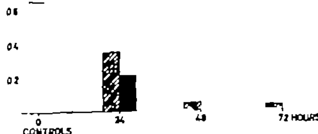


Fig 1 Heart content of NA after daily dose of reserpine of 0,05 mg/kg (striped columns) and 0.10 mg/kg (filled columns)

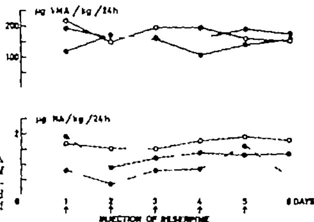


Fig. 2. Urinary excretion of VMA and NA after daily dose of reserpine of 0.05 mg/kg (semi-filled circles) 0.10 mg/kg (filled circles) and equivalent volume of drug solvent (open circles)

after reserpine treatment has been measured by Gaddum, Kr oy and La erty (1958). After a 15 mg njection they found a marked fall in the rate of excretion of NA. Similar results ha been reported by Carlsson, Boje Rasmussen and Kristjansen (1959) after prolonged reserpine treatment n man.

Mirkin (1961) observed moderate ncrease in the catecholamine excretion in the rat after administration of 1 mg/kg reserpine daily for 10 days. Hexamethonium lowered the excretion to bout one half of the normal. This alue was not further reduced by reserpine suggesting continued resynthesis even when the stores presumably are depleted. These results are in accordance w th those of Bucardi t al. (1964) who observed, after a single dose of 2 mg/kg reserpine in the rat, a fall in catecholamine excretion to bout one half of the original alue, even in adrenomedullectomized rats.

Excretion of NA in reserpine treated rats has been measured by Henning (1966). Injection of reserpine in a dose of 10 mg/kg depleted the tissues almost totally in 24 hrs, but rinary excretion of NA decreased only to about 20 per cent of the normal. After 48 72 hrs rinary NA had incr ased to about 50 per cent, but th tissues were st ll almost totall depleted.

In the prese t study reserpine treatment f r 5 days had no effect on urinary VMA. Normetanephrine nd 3-methox 4 h droxyphenyl glycol were not determined in the present tud and t can not be excluded that a larger proportion tha normal of these con pounds wer transformed to VMA as a result of reserpine tre ment. Assuming i addition, increased VMA formation as a result of n almost complet release of the t tal NA tores in the animal, which may be estimated t 0.1 µg g t the tot, or about 3 µg totally the VMA excretio would be increased by this amou t over a 48 hrs period. In both these ways a moderate reducti resynthesis migh be masked. However the maintenance of essentially norm

excretion values after reserpine in spite of severe depletion of the tissue stores of NA indicate that the mechanisms responsible for NA synthesis are far less sensitive to the inhibitory effect of reserpine than the uptake and storage system.

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Acta physiol. scand. 1968. 73. 527—535

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The "Diving Bradycardia" in Exercising Man

By

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Abstract

ASMUSSEN, E. and N.-G. KRISTIANSSON. *The "diving bradycardia" in exercising man*. Acta physiol. scand. 1968. *73*. 527—535.

The "face immersion reflex" (bradycardia on immersion of the face in water) was studied in normal young subjects, males and females, swimmers and non-swimmers, during rest [illegible] and during exercise on a bicycle-ergometer. During swimming in [illegible] steady state the heart rate was lower when the face was immersed while the subject breathed through [illegible] than when the face was out of the water at corresponding oxygen uptakes. During bicycle [illegible], apnea, with or without face immersion, caused bradycardia. [illegible] immersion [illegible] without apnea, caused only a short lasting, slight bradycardia that [illegible] on [illegible] face immersions. Varying the water temperatures between 15 and 35 [illegible] had very little eff[illegible] on the response to face immersion. The "face immersion brady[illegible]" in humans is [illegible] to be part of [illegible] conditioned reflex, developed from an unconditioned [illegible]

A pronounced bradycardia elicited by submersion in water has been demonstrated in various diving vertebrates, notably the seal (cf. Irving 193[illegible], Scholander 19[illegible]) and the duck (Paul Bert 1870, cited from Andersen 196[illegible]). It has been found re-peatedly also in humans (Scholander *et al.* 1962, Olsen *et al.* 1962, Irving 1963, Craig 1963, Hong *et al.* 1964) and it is suggested that it represents a part of [illegible] evolutionary old reflex—a general oxygen preserving reflex (Scholander 1963).

The stimuli responsible for the reflex bradycardia [illegible] in man may be several. Submersion of the whole body or immersion of the face (Irving [illegible] 1963) is normally accompanied by apnea and hence by hyper[illegible] and Scholander. The apnea may be accomplished with or without preceding hyper[illegible] the breath holding may proceed with the lungs in different states of inflation [illegible]. Intrathoracic pressure varies with the type of breath holding [illegible] tory alterations that reflexly influence the heart rate (cf. Craig 1963) [illegible] changed distribution of the blood between peripheral and central [illegible] that follows immersion [illegible] water influences the heart [illegible]. Further [illegible] of the water may play [illegible] role by eliciting reflexes, cutaneous [illegible] from the face. Actually cooling of the face alone by a [illegible] most the same effect as immersion of the face (Whayne and Killip [illegible]

Studies of the diving reflex in man have been performed with these considerations in mind and it emerges that the reflex bradycardia probably is composed of at least two reflexes, an "apnea reflex" and a "face immersion reflex" adding to or potentiating one another (Olsen *et al.* 1963, Craig 1963, Brick 1966, Paulev 1967).

It was originally stated that the bradycardia on immersion persisted in man in spite of "vigorous exercise" but rather few observations have been made during actual exercise. Scholander *et al.* (1962) studied male pearl divers during active apneic diving and compared their lowered heart rates (to about 50 beats per minute) with those obtaining during aerobic surface swimming. This latter activity was maintained for only about 1 min, and the pulse rates recorded were less than 120 beats per min. Hong *et al.* (1964) made comparable studies in Korean sea women professional skin divers. Their activities during surface swimming did not produce heart rates above 100 beats per minute and must also be termed short and light. Olsen *et al.* (196?) recorded bradycardia during underwater dives accompanied by "kicking". The period of activity was also here of low intensity and restricted to one to two minutes even after vigorous hyperventilation. Craig (1963) reports one experiment in which his subject—during an apneic dive after hyperventilation in 100 % O_2—exercised by moving his flippers up and down at a moderate rate and force for 35 sec. This increased the heart rate from about 50 to about 75 beats per minute.

It seems therefore that the claim that "the bradycardia persists in spite of vigorous exercise as is the case in diving vertebrates" (Andersen 1966) rests on rather weak evidence. The exercise studied has been very short lasting, never reaching a steady state; its intensity has not been measured, but judging from the recorded pulse rates, hardly been what might be called "vigorous".

present investigations were undertaken with the purpose of studying 1) the face immersion on heart rate in the steady state of swimming at known energy outputs (oxygen uptakes) and 2) the effect of apnea and face immersion, separately and combined, on the heart rate during steady states of exercise of known intensities on a bicycle ergometer. As subjects served young (17—28 years) male and female volunteers. In the first series only one subject took part. He was a well trained, fairly accomplished swimmer. In the second series 6 subjects were studied. *One of these (male) was an untrained non-swimmer. Two others (one male, one female) were in good physical condition, but only occasional swimmers.* Three—one female and two males—were competitive swimmers in excellent form.

Methods

Heart rates were recorded by means of electrodes pasted to the thorax and connected to an amplifier. During swimming the heart beats were counted from the clicks of the recorder over periods of 30 sec. The counting was repeated 2 to 3 times to ensure that steady state had been reached. In the bicycling experiments the heart beats were registered continuously on an ink-writing recorder and subsequently counted in periods of 4 sec and converted to beats per minute. Work intensities were in the swimming experiments expressed by the oxygen uptake. The subject (one only) breathed through mouthpiece and light-weight plastic valve. The inspiratory and expiratory tubes were prolonged and extended backwards over the subject

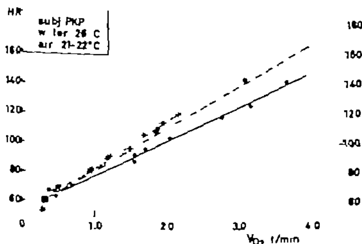


Fig. 1 Heart rate in relation to oxygen uptake in PKP (ca. 25 years) during supine bi y ling (— +) and during swimming with face out of (○———○) or in ter (●———●)

head in such y that he could swim (breast-stroke) either with the face abov w ter or ith the face immersed, w th normal respiration. The expiration tube was connected through flexible, ide-bore rubber tubes and three-way stop-cock t Douglas bag for collecting of expired air. The Douglas bag and the amplifier for pulse recording ere carried by the ex perimenter who followed the swimmer walking at the side of the indoor swimming pool. Gas volumes ere measured by means of calibrated, 120 l gas meter. Gas analyses were per formed (in duplicate) on the Scholander gas-analyzer.

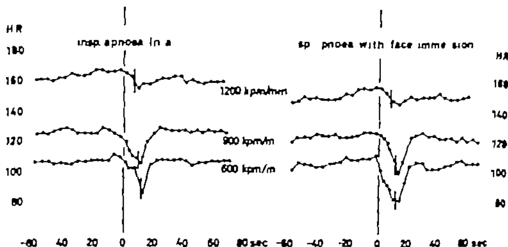
The bicy le experiments ere performed on Krogh-bicy le ergomete which permits an accurate measurement of he external work in kpm/min. The subjects rode in forward inclined position, the handle bars being placed in the same as as in bicy le-racing. Face immersion could therefore be accomplished by hoisting plastic container filled ith water p under the head of he subjec withou hange of position. He was in all experiments breathing through mou h piec and alve of the same kind as used in he swimming experiments. I one series h the first subject) bicycling was performed ith the subjec lying on his back and w th the legs horizontal, to liminate he effect of grav on the circulation. I this series the oxygen ptake was determined as in he swimming experiments.

Results

In Fig 1 ar plotted the heart rates agains the oxygen ptakes n l m n f om tw series of wim ing experiments. In one series the subject PKP m le 5 ears kept his face out of the water in he other also his face was immersed. F r mparison the results from the supine bicycling experiment ar includ d. Sw ming w th the face out of the water ga slightly lower pulse f equ n than supine bic ling in air at corresponding oxyge ptakes. W th th f mersed during w mming there was f rther lowering of the pulse rate V $_{O_2}$ lation. Th e rates in the resting stat did not show m corresponding d fferences.

I the bicy ling experiments the subje ts w tested

These expe ments, and he swimm g experime ts, er j of h...



F g. 2. Heart rates t three levels of steady state bicycle riding, with periods of inspiratory pnea, without and with face immersion (HHJ m. 28 years, non-swimmer)

cise, *giving steady state heart rates of approximately 100, 120 and* 150—160 beats per minute. The tests consisted in 1) apnea in near maximal inspiration, 2) apnea in inspiration with face immersion (Fig. 2) 3) apnea in expiration with face immersion (Fig 3) and 4) face immersion without breath holding The subjects were instructed and trained in keeping the glottis open during breath holding The male subject was well trained accustomed to the experimental procedure but was not a swimmer

piratory apnea induced a bradycardia that became gradually more pronounced the duration of breath holding. Breath hold times were shorter the heavier the load and the degree of bradycardia, probably for this reason, was smaller uring heavy exercise than during light or moderate exercise. Th addition of face immersion (water temp. 25 C) had only a slight effect on the bradycardia (Fig. 2)

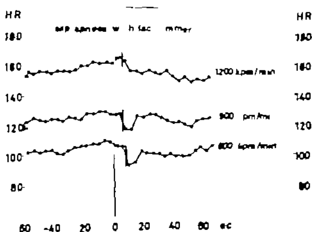


Fig. 3 As in Fig. 2 but ith expiratory apnea.

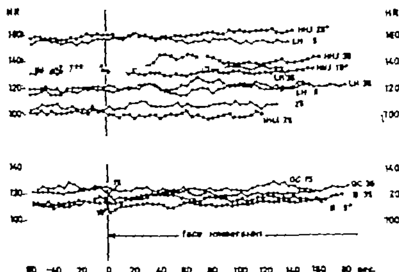


Fig. 4. Heart rates during steady states of exercise, with face immersion from time 0. Upper group of curves from two well trained non-swimmers (HHJ m., and LH fem.) lower group from two competition swimmers (OC, m. and NB m. The temperature of the w ter is given in C.

Expiratory apnea w th face immersion (Fig. 3) had in this subject ery little effect on the heart rate during the actual breath holding but he and the other subjects showed a continued decrease of the heart rate during the first 5 to 10 sec after the apneic period.

Face immersion alone without breath holding (condition 4) had practically no effect on the heart rate t w t r temperatures 25 and 35 in any of the subjects (Fig. 4). At water temper t res of 15 the most skilled subjects one male, one female well trained non- wimmers showed no brady ardia at face immersion. Of 4 other subjects, less well n ed to the procedure, 1 non-swimmer and highly trained swimmers showed a slight brady ardia on face immersion in cold water (15 C). It disappeared however during the following 20 to 40 sec. One wimmer showed no brad cardia on t mmersio cold water. Examples on the two skilled subjects and on two wimmers re show n F g. 4.

In the experiments w th p c t immersio n F g. 2 and 3 the breath holding times were predetermined as not t use t great disc mfort to the subjects. With the trained swimmers series of experiments were performed which the breath holding was continued for as long as possible. Two examples of such experiments with face immersion are shown in F g. 5, both w th inspiratory apnea and with expiratory pnea. The bradycardia which onl was small when the breath holding time was short, here developed further in on subject d ring expirato ry apnea to the extremely low heart rate of 10 beats per minut The decr se

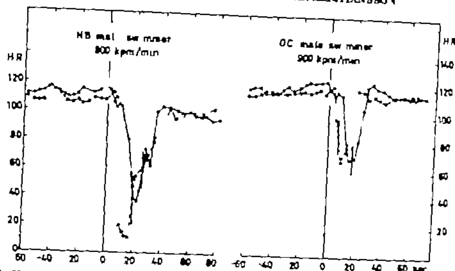


Fig. 5. Heart rates in the steady state of exercise on two male swimmers, with maximal apnea under face immersion, in inspiration (black dots, full lines) and in expiration (open circles, dashed lines).

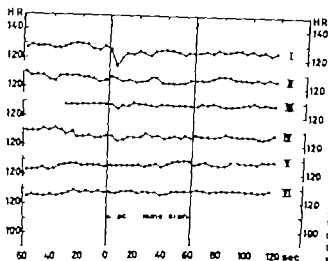


Fig. 6. Heart rates during uninterrupted exercise with face immersion (5 C) repeated 6 times at 5 min intervals (male swimmer).

to proceed faster during expiratory apnea than during inspiratory apnea. Fig. 6 shows as an example the heart rates in an excellent swimmer during face immersion without breath holding, repeated 6 times during uninterrupted exercise at intervals of about 5 min. Only the first face immersion produced a passing bradycardia.

Discussion

Our bicycle experiments have confirmed that voluntary apnea, also in the steady state of exercise, produces bradycardia. Apnea, combined with face immersion, produces a bradycardia that is only slightly more pronounced than apnea in air. The degree of pulse slowing depends on the length of the breath holding time. Probably

for this reason the heart rate decreased less during hea y wo k, when the eath hold time was shortened than during light work, and likewise less during expir tory apnea than during inspiratory apnea. Face immersion al ne however (Fig. 4) had only a slight or no effect on the heart rate which soon returned to the steady state value. The effect of face immersion depended only little on the temperature of the ater (15 25 and 35 C) There was a tendency for the first experience of immersion to produce the most pronounced pulse slowing independently of the water temperature. In all breath holding experiments, w th and without face immersion large individual differences occurred, and even the same indi idual could, on dif ferent days under apparently identical conditions, show large differences in his or her response to apnea.

Our results with simulated diving during the steady state of exercise are not principally different from those of other investigators of the diving reflex in man (g. Scholander *et al.* 1962, Olsen *et al.* 1962, Irving 1963 Craig 1963 Hong t *al* 1964) Common is that the bradycardia in man develops gradually w th time of apnea, in our experiments usually with a latency of 5 to 10 sec. Th return to pre di e alues is also delayed so that often the lowest heart rates are found after the apnea. This time-course is different from that found n a typical di ing animal as the harbour seal (Scholander 1940) where s bmersion of the nose immediately elicits a lengthening of the very first pulse interval, but it resembles that seen for instance in the domestic duck (Andersen 1963)

In the duck the diving reflex is elicited from receptors in the beak and mediated through the trigeminal nerv (Andersen 1963) The bradycardia on submersion of the nostrils was somewhat reduced but not eliminated if the duck s lungs were ventilated during submersion (Feigl and Folkow 1963)

In resting man face immersion without apnea was found by Brick (1966) nd Whayne and Killip (1967) to cause a slowing of the resting heart rat In the present work experiments face immersion alone w thout pnea caused no brady cardia in the 2 subjects (non-swimmers, but in good physical cond tion) that were best acclimated to the experimental procedure (Fig 4) In the less well acclimated swimmers and in one untrained non-swimmer face immersion caused passing slight bradycardia, especially in the first experiments and cold w ter (Fig 4 W found that on repetition of the face immersio the response diminished nd disappe red. This is demonstrated in Fig. 6. If fac im rsion brad cardia is reflex, it seems to be rather inconstant and fleeting one The possibility th t t is conditioned reflex, developed in swimmers, seems quite prob bl (f Irving 1963

The diving bradycardia in man, consequ ntl seems mainl to be an pnea bradycardia probably part of some ther well documented circulatory responses to apnea (f Olsen t *al* 1962, Brick 1966) Ther sev al possible explanations for the pnea bradycardia on submersion (Craig 1963 based n ts dependence on the degree of lung inflation, the intra-thoracic pressu res arterial h poxia and hyper capnia, the re-distribution of blood from periphery to entral essels because of the elimination of the ffect of gra ty on the blood and of cold on the filli g

cutaneous veins. None of these seem completely to fit the present experiments, in which the apnea bradycardia was relatively independent of whether apnea was maintained in inspiration or in expiration, no Valsalva-like manoeuvers were performed, a hydrostatic effect was eliminated, and in which the temperature effect was negligible. Hypoxia and hypercapnia generally cause tachycardia. One fact seems to be noteworthy During apnea a certain increasing inhibition of reflex breathing must take place. In experiments like the present, this inhibition is voluntary probably cerebral of origin. In other cases—in diving animals, in drowning animals—the respiratory inhibition may be reflex, elicited from receptors in the face or nostrils. This respiratory inhibition gradually may spread to other centres, thus causing the circulatory changes, including the bradycardia that follows apnea. From a teleological point of view this explanation seems plausible Respiratory arrest on face immersion must be immediate to be useful, whereas the circulatory changes, beneficial as they may be especially in diving animals, seem less urgent. An irradiation of the inhibitory state may develop into a conditioned reflex, selectively strong in diving animals, elicited from sensory receptors in the face or around the nostrils. In this connection it may be mentioned that Andersen (1966) cites reports to the effect that the diving bradycardia is less well developed in seal pups than in adult seals, and that both seals and ducks improve their diving endurance with training.

The bradycardia seen in human swimmers, also on face immersion alone, may then likewise be taken to be a conditioned reflex. In the case of face immersion without apnea it seems to be easy to extinguish (Fig 6) With the whole body in water as during actual swimming and diving it may be more persistent. Therefore, the lowered heart rate V_{O_2} relation during swimming with snorkel and face immer (F g 1) may possibly be due to a conditioned reflex. The low heart rates during ic diving seen in pearl di ers, sea women and other professional skindivers then be a combination of a conditioned face immersion reflex and an irradiated inhibition of the circulatory functions, caused by the pnea

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From the Research Laboratories of the Stat Alcohol Monopoly (Alk) Helsinki, Finland

On the Interconversions of Amino Acids of Brain in vitro with Reference to the Effect of Ethanol

By

Hertta-Maija Hakkinen and E. Kulonen

Recei ed 11 January 1968

Abstract

Hakkinen, H. M. and E. Kulonen. *On th int conversions of amino acids of brain in vitro with referenc to th ff ct of ethanol* Acta physiol. scand. 1968. 73 536—542.

GABA was converted glutamic acid and in arying degree to glutamine during incubation with brain preparations. Aspartic acid was formed mainly from glutamic acid. It is concluded that ethanol, when given situ disturbs both the catabolism of GABA and the synthesis of gl tamine from glutamic acid in the brain

I the brain homogenate nearly all the GOT activity was found in the postmitochondrial supernatant, equally distributed bet een the postmicrosomal supernatant and microsomal particles The GPT acti ty was much lower and the microsomal particles ere devoid of it. After stor ge at −18 C the GPT activity in the preparations from ethanol-treated rats de reased significantly more pidl than in orresponding preparations from control rats.

previous studies n i o we found that the concentrations of certain amino acids (GABA glutamic acid and aspartic acid) are increased in the brains of ethanol-treated rats and that the concentration of glutamine is decreased (Hakkinen and Kulonen 1961 From further experiments w th electricall stimulated slices we deduced that the ncrease of GABA depends on diminished breakdown, but the changes in glutamine were more difficult to interpret (Hakkinen, Kulonen and Wallgren 1963)

The experiments reported here wer undertaken to clarify the mechanism of these ethanol-induced changes Studies on the interconversions of th amino acids mentioned above and on their transamination were considered relevant.

Experimental

Brai pr parations. Rats of the Wista trai weighing 190— 40 g had fasted for about 40 hrs before the experiments. As pretreatment n 50 mg of ethanol pe 100 g of b t. as given as a 33 per cent (/v) aqueous sol tion by stomach tube The control animals received an equal olume of water The rats were killed by dec pitation after 1 hr The brains ere

Abbreviations GABA = γ-aminobutyric acid GOT = aspartate aminotransferase (EC 2.6 1 1) GPT = alanine aminotransferase (EC 2.6 1 2)

dissected out immediately at room temper ture, eighed on torsion balance and homogenized ith a Potter-Elvehjem pparatus in 4-fold olume (/w) of cold or om potassium chloride-potassium phosphate solution, pH 7 4 (DiPietro and Weinhouse 1959 The subcellular fractions (Table III) ere obtained by differential centrifugation at 0 (From the supernatant obtained on entrifugation t 100,000 g for 60 min the soluble protei fraction was separated by gel filtration through Sephadex G-25 column (Flodin 196

The incubation medium as adopted from DiPietro and Weinhouse (1959) and the con ditions of incubation as explained in the legends to the tables and figures.

Determination of ami acids. I the experiments with hole brain homogenate (Table I the amino acids in the incubation mixture ere determined by a paper-chromatographic method (Hikkinen Kulonen and Wallgren 1963) I the experiments ith the postmito-chondrial supernatant and soluble protein f action (Table II and Fig. 1) the amino acids were analyzed by the paper-electrophoretic method described by Hakkinen and Kulonen (1963)

Determinations of GPT and GOT. The activities of GPT and GOT in the brain homogenate ere determined according to Reitman and Frankel (1957) following the directions given in the leaflet published by C. F Boehringer & Soehne G.m.b.H., Mannheim, Germany for the use of their reagent packages (TC-R/S 964 3 Auflage, 1962) The results were calculated in International enzyme units per g of original brain, using the data given by the manufacturer The suitable amounts of brain homogenate er found in preliminary experiments (for GPT test 0 1 ml of homogenate, corresponding to 20 mg of brain, and for GOT test 100-fold dilution, corresponding to 0 2 mg of brain) The determinations were made in duplicate at least.

Results

C nversion of GABA and glutam acid to th am n acid In incubated b a n hom genat roughly equal amounts of added GABA are converted nto glutamine and glutamic acid (Table I) but much less to aspartic acid and alanine Add tion of glutamic acid increases the oncentration of glutamine to almost 5-fold, and that of GABA, aspartic acid and alanine to about 2 fold. Aspartic acid is thus derived from glutamic acid rather than from GABA Glutamic acid i also a more significant donor of ammonia to pyru ate than GABA

TABLE I Con ersion of GABA and glutamic acid to other mino cids on ncuba ion of brain homogenate

The incubation mixture (2 5 ml) contained he sample corresponding to 60 mg of brain and 10 mM glucose, 0 1 mM fumarate 8 mM $MgSO_4$ 77 mM KCl nd 40 mM Na_2HPO_4-NaH_2PO_4 buffer pH 7 4. The incubation ime as 1 hr and empera ur 37 5 C GABA as added, 20 8 mmoles, and glutamic acid, 40 mmoles per 100 g f brai The figures erages f duplic te incubations ith pooled samples from s The alues are calculated in moles per 100 g of original tissue

Amino acid determined	No additions	GABA added	Glutamic acid added
	Concn	N increments	
GABA	430		360
Glutamine	350	840	890
Glutamic acid	910	40	
Aspartic acid	330	130	470
Alanine	320	80	360

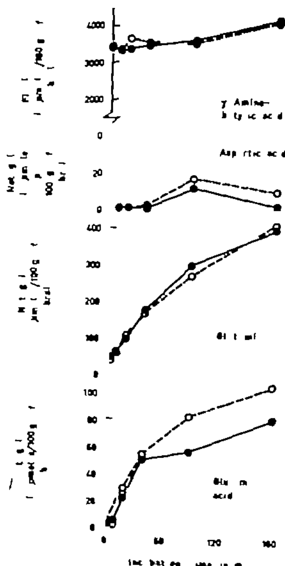


Fig. 1. The effect of incubation time on the conversion of GABA to other amino acids by the soluble protein fraction of brain. The incubation mixture (4.0 ml) contained the sample corresponding to 500 mg of brain and 10 mM glucose, 0.1 mM fumarate, 8 mM $MgSO_4$, 77 mM KCl, 2 mM NAD, 0.06 mM cytochrome c, 1.4 mM ATP and 40 mM Na_2HPO_4—NaH_2PO_4 buffer, pH 7.4. The temperature was +37.5 °C. GABA was added, 4600 μmoles per 100 g of brain. Pooled samples from 3 ethanol-treated (○- - -○) and 3 control (●——●) rats were used, and the incubations made in duplicate.

Corresponding incubations were also performed with the *postmitochondrial supernatant*. It was confirmed that aspartic acid is formed from glutamic acid but not from GABA. With this preparation more glutamic acid than glutamine was formed from GABA. Ethanol decreased the synthesis of glutamine from glutamic acid from 3470 to 3300 μmoles per 100 g of brain and increased the synthesis of aspartic acid from 670 to 710 μmoles per 100 g of brain, which changes are in agreement with the previous work.

In Fig. 1 are shown the corresponding data on the metabolism of GABA in the incubated *soluble protein fraction*. The relative constancy of the concentration of GABA is noticeable. More glutamine than glutamic acid is formed from GABA, but

TABLE II Mutual con ersions of amino acids by incubation with the soluble protein fraction of brain homogenates from ethanol-treated and control rats

A sample of the soluble protein fraction corresponding to 200 mg of brain tissue was incubated for 1 hr in the medium described in the legend to Fig 1 Each amino acid was added t rate of 3440 μmoles per 100 g of brain. Pooled brain homogenates from 2 ethanol treated and 2 control rats ere used in preparation of the samples, which were incuba ed in duplica e. The effect of ethanol is expressed in per cent of the corresponding alue in control experimen

Amino acid added	Treatment of rats	Final concentration in μmoles/100 g or original brain			
		GABA	Glutamine	Glutamic acid	Aspartic acid
GABA	W ter	1100	240	10	0
	Ethanol	+ 7 8	— 4.2 *	—	—
Glutamine	W ter	170	1600	90	0
	Ethanol	+16.7	+ 7.6 *	+36.8	—
Glutamic acid	Water	190	600	2330	20
	Ethanol	+11.5	— 7 1	— 4.7 *	+50.0 *
Aspartic acid	Water	90	160	80	1950
	Ethanol	+11.8 *	— 2.7	+37 1	— 0 4 *

single incubation only

very little aspartic acid. The formation of glutamine is almost linearly correlated w th the duration f incubation, and it seems not to be affected by ethanol. The expected ffect of ethanol is noted the formation of aspartic acid and glutamic acid.

These data are further extended in Table II w th pecial reference to the effect of ethanol. In these conditions only gl tamine is prod ced by th dd tion of GABA GABA and some gl tamic acid are formed from gl tamine Gl tamic acid is con erted to gl tamine nd to lesser ext nt to GABA

Ethanol causes decreased loss of added GABA, which support the iew that th *increased* *ncentration of GABA in th br ins of ethanol treated rats depend on* disturbed atabolism (Hakkinen, K lonen nd Wallgren 1 65 All th alues of glutamine nthesized from other amino acids re decre sed by pretreatmen w th ethanol, as expected The breakdown of added l t ne is als decreased by ethanol Both the synthesis and breakdow of gl tamir t ppe t be af fected by ethanol

T an minase The postmitochondrial s pernatant is relati ely ric in both GOT and GPT (Table III The microsomal sediment contains nsiderable GOT acti t when tested immediately but is devoid of GPT Ethanol enhanced he GOT activit slightl in ll the homogenate fract ons This effect of ethanol was more marked after storage of the postmitochondrial supernatant f r 15 days t — 18 C.

TABLE III Alanine aminotransferase (GPT) and aspartat aminotransferase (GOT) in subcellular fractions of brain homogenat

The postmitochondrial supernatant was obtained by centrifugation t 5000 g for 10 min and the postmicrosomal supernatant at 100,000 g for 60 min. Samples from 2 ethanol-treated and 2 control rats wer pooled, and the determinations were made in d plicat

Fraction of homogenat	Treatment of rats	International units per g or of original brain	
		GPT	GOT
Whole	W ter	1.56	113
	Ethanol	1.50	128
Postmitochondrial supernatant	Water	2 19	106
	Ethanol	2.17	119*
Postmicrosomal supernatant	W ter	0.85	59
	Ethanol	0.86	65
Microsomal sediment	W ter	< 0 03	51
	Ethanol	< 0 03	62

after storage the values are 92 and 130, respectively
decreases t zer during storage for 15 days at − 18 C

These experiments were repeated ith six ethanol intoxicated and six control rats to obtain more information on the effect of storage (Fig 2) In the preparations from ethanol treated rats the GPT activities decreased significantly faster than the corresponding values of the control rats. The GOT activities were higher in samples ethanol-treated rats than in those from control rats, but the differences were nificant except after storage for 2 weeks (P<0 05)

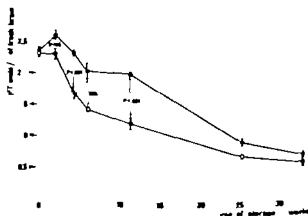


Fig. 2. The effect of storage at −18 C on the GPT activit es of brain homogenates from ethanol-treated (O———O) and cont ol (● – – – ●) rats The standard errors of the means and the statistically significant differences are ndicated (=6) The tests ere performed with postmitochondrial supernatants (see the legend to Table III) which were briefl thawed for each determination to measure be liquots for the analyses

Analogous experiments were performed by addition of ethanol to the p chondrial supernatant *in vit o* The amount of ethanol added (5.3 mg pe postmitochondrial supernatant) was calculated to approximate to the maxi centration in the brain when ethanol had been administered *in vivo* In th e periments likewise, ethanol accelerated the decrease in GPT acti ty After t for 4 months at − 18 C without intervening thawing, the decrease in GPT act was 46 per cent in control samples, 55 per cent when ethanol had been gi n *vi* *vo* and 65 per cent when it had been added *in vitr*

Discussion

The increases in GABA, glutamic acid and aspartic acid and the decrease in gl t amine, which are observed as effects of ethanol (Hakkinen, Kulonen and Wallg n 1963) cannot be explained by any altered activities of alanine or aspartate aminotransferases. Only during storage does the presence of ethanol cause significant inactivation of alanine aminotransferase.

The apparent constancy of the GABA concentration during incubation (F g 1) is noteworthy Equilibrium states seem to be formed between GABA nd it de gradation products. The level of GABA depends on the brain preparation, on pre treatment with ethanol and on the other experimental conditions. Although it seem established that ethanol affects the level of GABA by its ction on catabolism, we cannot concl de whether it is the transamination to succinic semialdehyde or the subsequent oxidation to succinic acid that is dist rbed

A natural explanation of th effect of ethanol on the levels of GABA and glutamine would be that the formation of gl tamine from GABA is dist bed. The present results do not support such a hypothesis (Fig 1) Rather it an be stated that ethanol affects th ynthesis of gl tamine from gl tamic acid (T ble II) as has been demonstrated directly by the determination of gl tamine ynthetase activ ty (unpublished work

When the samples f om ethanol-treated rat are f oze t low t mperature ethanol is concentrated the liquid phase For example t − 18) C the liquid phase contains 29 9 g of ethanol per 100 g of the satu ted aqueous solution (Seidell 1941) which ma nact the enzy ies es t low tempe t e. During storage of biological samples such ncreased conce tr t on of ol may be source of artefacts

The detrimental ffect of pretre tment w th ethanol vo on the tability of the enzyme activities of the brai prepa t on ay l lated to the observ tion that during aging the brain preparations f om etha l t eated rats lose the bility t mediate the cha acteristic ffect of ethanol (Hakk nen and Kulonen 1967) On th other h nd, in the preparat ons fro cont l ts the same changes In etal l capac ties occurred during ag ge wer used immediatel by ethanol ment.

We suggest that ethanol influences the molecular organization of the nervous tissue which becomes more labile and is more easily changed to metabolically different forms. Several functions may be affected simultaneously and to varying extents.

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From the Department of Neurology Karolinska Sjukhuset, Stockholm, Sweden

Properties of Motor Units in the Rat Anterior Tibial Muscle

By

Lars Edström and Eric Kugelberg

The anterior tibial muscle of the albino rat shows a mixture of enzymatically distinct muscle fibres. F om the standpoint of succinic dehydrogenase activity 3 different types of fibre have been distinguished the classical white muscle fibres (A fibres) and 2 types of red fibres (B and C) (Stein and Padykula 1962)

It has recently been shown that muscle contractions produce histochemical changes in the phosphorylase activity and glycogen content which [illegible] aried in the different types of fibre (Kugelberg and Edström 1968 a and b The distribution of histochemical effects in the muscle on stimulation of a single motor nerv fibre has been used to elucidate the histochemical omposition of the mot r nit the spatial arrangement of its fibres nd the orrelation between it histochemical and f nc tional properties. These ha been investigated earlier by les direct methods g Krnjević and Miledi 1958 Olson and Sw tt 1966 Close 196 nd ther for re quir further tudy heterogeneous muscl

It was found that he histochemical omposition f f b es thi ngle motor unit was largely n form Fig 1 Seven motor un h re ng of about 100 fibres wer in estigated Fou were omposed m t t I d A fibres 0—5 were B fib es and none C fibres Three wer om p sed lm t tirely of B f bres only a few pical A fibres were observed

The f bres t single motor uni wer s tt ed 20—25 of the cross sectional re of the m scle generall lon bu ome unes n groups of —4 f bres Thus the es ensi overlapping t d ni The fibre density w largest n the en f the territory of the units

The A nit wer f st th on ac me of about 13 msec nd fatigued rapidl even t low frequency timula on 10 sec The B un t had a contraction time of bout 13 msec nd showed much les tendency to f tigue C nits showed no evidenc of f tigue

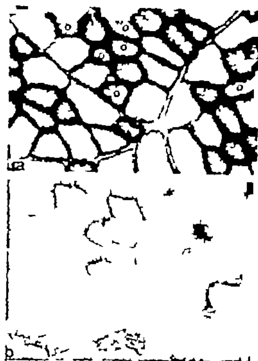


Fig. 1. Serial sections of anterior tibial muscle incubated for ( ) succinic dehydrogenase and (b) PAS. × 120.
The PAS negative fibres represent small part of a single motor unit of about 100 fibres. All fibres show similar intensity of succinic dehydrogenase activity (B fibres). The PAS negative fibres in b are in indicated with an open circle.

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D 1

Department of Physiology, University of Göteborg, Sweden

Input amplifier for intracellular potential and conductance measurements

By

E. Eide

The amplifier allows recording of potentials produced across the cell membrane by current pulses injected through the recording microelectrode.

Components inside the dotted line (Fig. 1 A) are housed in a small box ($30 \times 40 \times 15$ mm) directly attached to the manipulator. The input and the feedback-compensation tubes are nuvistors 8056, giving low microphony. The tubes are operated with constant cathode current. Anode voltage is driven from the cathode via a

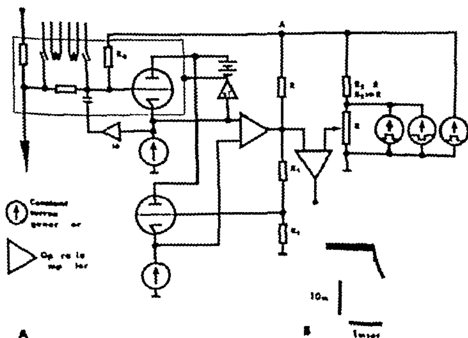


Fig. 1. A [illegible] text. B shows the onset of the response from a current pulse of 16 nA [illegible] motoneurone recorded with KCl-filled microelectrode of 6 MΩ, inserted [illegible] cord to depth of about 2 mm.

unity gain amplifier Thus no change occurs in the tube parameters with input voltage changes, and the cathode follows the grid with unity gain. The DC difference between the grid and the cathode of the input tube is compensated by the feedback-compensation tube.

Current pulses can be applied to the impaled neurones through a resistance R_0 of 10 MΩ For correct compensation of the voltage drop in the microelectrode resistance, the time constant of the pulse as well as the amplifier must be very short compared with the time constant of the cell membrane. A generator essentially operating from point A with unity gain with respect to the input is obtained by mixing the outputs from the ×2 amplifier and the pulse generators in a resistance network R_2 a. The current will have a short time constant because of the low value of the resistance R_0, and is independent of voltage changes at the input. At the same time input resistance is high. The voltage drop in the electrode resistance caused by the current pulse is compensated by subtraction from the ×2 signal in a differential amplifier with a gain of 5 The result is an exact reproduction of the cell membrane voltage event, 10 × amplified. Input capacitance is neutralized in the normal way with positive feedback.

D 2

Department of Physiology University of Goteborg, Sweden

Remotely controlled micromanipulator for neurophysiological use

By

E. Eide and Y Kallström

A remotely controled manipulator has been constructed, based on the usual manipulator principle of a micrometer screw

The micrometer screw is driven from an electronically controlled stepping motor making one complete revolution upon 48 step impulses. The transmission from the motor axle gives a discrete final electrode movement of 2μ upon each pulse. Another synchronously driven stepping motor operates an indicator clock. With this arrangement the accuracy is dependent only on the precision of the mechanical transmission (Fig 1)

The electronic drive unit provides different modes of operation

1 *Continuous* A continuous train of step impulses, with selected intervals of 5, 10, 40 and 200 msec, are fed to the motors as long as the up or down button is pressed.
2. *Single* A preselected number of pulses (1—10 with the selected intervals) follows each button activation
3 *Externally triggered* A preselected number of steps follows each external triggering pulse with adjustable delay as long as the button is pressed.

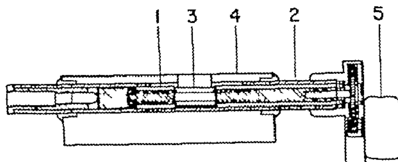


Fig. 1 The figure show longitudinal section of the nee omanipulator The micrometer screw (1) is spring loaded and mounted in ball bearings in tubular slide (2) thus minimizing back lash in the manipulator A tab on the micrometer nut (3) protrudes through a slit in the slide and is fixed in the manipulator housing (4) The stepping motor (5) mounted on the top of the tubular slide, is geared to the screw in one step with gear ratio of 12 125 The maximal length of remotely controlled movement is dependen on the length of the micrometer screw Both 25 and 60 mm stroke lengths ha been used. Electronic end stops limit the movement t either end of the micrometer screw

The normal procedure is to approach the tissue w th a coarse manual rack and pinion movement. Then, the continuous mod of remot micromanipulator control to contact the microelectrode tip with the tissu and to drive the electrode to the region under investigation The external triggered mode linked to the CRO sweep n then used to impale the neurones At this tage it is often fa ourable to use short trains of $5 \times 2\mu$ advanc (n contrast to the smooth mo ement of the usual types of manually operated manipulators)

Ten manipulators ha been employed n neu oph siological research for some years and hav proved to be reliable n ement nd to facilitate microelectrode impalement of neu ones

Because of the digital princ pl of oper tio n t is be possible to use an electronical bidirectional ounte d pth indic t r n tead of the step motor driven indicator clock. With this t pe i ndic or h posit on of the electrode may be read in digital form

D 3

Dep t Ph siolog U set of Goteborg, Sweden

Differential chloride reversal of IPSPs from group Ia afferents and motor axon collaterals

By

R E Bu rk I Fedina and A. Lundberg

Micro k ph resis je on of hloride ions (Cl) nto spinal motoneurones causes IPSP from rce of afferent sources to reverse from hyperpolarizing to depolar ng potential due to modification of the IPSP ionic equilibrium pot (Coom l et l 1955 Araki et al 1961)

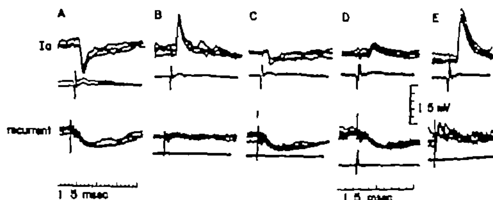


Fig. 1 Records from a posterior biceps-semitendinosus motoneurone with stable membrane potential of 58 mV KCl electrode. *A* Direct group Ia IPSP from quadriceps nerv (upper records) and recurrent IPSP (lower records) immediately after cell penetration. *B*. Reversal of both IPSPs during steady cathodal (hyperpolarizing) current of 15×10 A passed through the microelectrode. *C* Immediately after current turned off *D* About 15 min after *C*, Cl leakage reversed the direct but not the recurrent IPSP *E*. Immediately after *D* cathodal current of 10×10 A causes more effective reversal of both IPSPs than in B. Calibration: 5 mV for all records 1+5 msec (records in *D* and *E* on faster time base)

We have observed that with relatively small Cl injections into cat spinal motoneurones, the time course of IPSP reversal and subsequent recovery often may be different when the IPSP evoked from volleys in antagonist group Ia afferents was compared simultaneously with the recurrent IPSP generated by antidromic volleys in motor axon recurrent collaterals. Small increases in intracellular Cl concentration had an apparently greater effect on the group Ia IPSP than on the recurrent IPSP (Fig 1 A—C) and it was frequently possible to reach a point at which the up Ia IPSP was completely reversed to a depolarizing potential while the recurrent IPSP remained, or had returned to, a hyperpolarizing transient (Fig. 1 D). Qualitatively similar differential Cl reversal was seen in 20 of 23 motoneurones studied with KCl filled microelectrodes. The apparent degree of differential reversal varied from cell to cell and in 3 of the 23 cells, the reversal of Ia and recurrent IPSP with Cl was quite parallel.

It is postulated that the synapses generating the recurrent IPSP may be situated on dendritic membrane regions relatively more distant from the site of ion injection (presumably the cell soma) than are the synapses activated by the group Ia inhibitory pathway The available evidence suggests that the inhibitory synapses activated by antagonist group Ia fibres are located largely on the motoneurone soma while those of the recurrent inhibitory pathway (from Renshaw cells) are largely on the proximal dendritic branches.

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D 4

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Vibration receptors in the forelimb of the cat

By

H. Silfvenius

When investigating the cortical projections of large muscle spindle afferents from the forelimb, the problem arose, whether the observed responses were evoked by these afferents only or whether afferents from Vater-Pacinian corpuscles contributed. Both receptor types may respond to vibrating stimuli (Hunt and McIntyre 1960 Brown, Engberg and Matthews 1967) and both have fast conducting fibers with low threshold to electrical stimulation. It was therefore decided to compare the cortical responses evoked by a nerve from Vater-Pacinian corpuscles with those of a muscle nerve. The interosseous nerve in the hindlimb described by Hunt and McIntyre (1960) offers a sample of afferents from Vater Pacinian receptors. Such receptors located in the forelimb were described by Skoglund (1960). The course and the conduction properties of the afferent fibers from these receptors were studied in the present report.

The *radial-ulnar* interosseous membrane is covered with clusters of Vater-Pacinian corpuscles forming an organ suitable for the reception of vibration stimuli. The afferent nerve is a branch of N. medianus given off distally to the branches

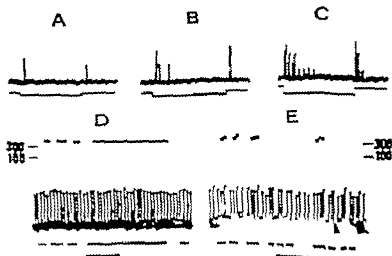


Fig. 1. A–C. Afferent impulses recorded from a thin strand of the forelimb interosseous nerve. Stimulation with square mechanical pulse applied to cluster of Vater-Pacinian corpuscles. Pulse amplitude A, B, C. Pulse duration A 37, B 6, C 130 msec. D–E Frequency response of receptor. Upper tracing: Spike interval recorder, calibration imp. per sec. Middle tracing: Action potentials. Lower tracing: Counted spikes. Time 20. Spikes counted.

for m. pronator teres, m. flexor carpi radialis and m. flexor digitorum profundus. It dives deep between the two last mentioned muscles and approaches the interosseous membrane. The axons are distributed to about 30—40 typical Vater Pacinian corpuscles. The receptors are sensitive to mechanical stimulation, fast adapting and follow vibrating mechanical stimulation to 500 per sec (Fig. 1) The nerve contains about 200 myelinated fibers with diameters between 15 and 2 μ 44 per cent of them are larger than 9 μ The conduction velocity of the fastest afferents varies between 130 and 90 m per sec. The compound action potential of the afferent nerve displays a delta component with a conduction velocity of 30—15 m per sec 28 per cent of the axons have diameters between 5 and 2 μ

Investigations of the cortical projections of these afferents are in progress.

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D 5

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Use of partial α receptor blockade for estimation of transmitter concentration at vasoconstrictor nerve endings

By

B. Ljung

Comparisons of smooth muscle responses of isolated vessels to administration of noradrenaline (NA) in the bath and to constrictor nerve stimulation, respectively cannot be directly utilized for quantitative estimations of the transmitter amounts released since 1 all the muscle cells are not innervated and 2. myogenic propagation contributes to the neurogenic responses. (Johansson and Ljung 1968)

However partial blockade of the adrenergic α-receptors by phenoxybenzamine might allow an approximate estimation of the transmitter concentration in the neuro-muscular gap during sympathetic stimulation. This was attempted in an in vitro study using a nerve muscle preparation consisting of the rat portal vein and its postganglionic nerve supply

Fig. 1 A illustrates the results of an experiment, where the individual responses to increasing concentrations of NA and to nerve stimulation after a partial α-receptor blockade (phenoxybenzamine 10 moles/l) and total β-receptor blockade have been expressed as percent of the corresponding responses before the α-blockade. Evidently the degree of reduction of NA responses is inversely proportional to the NA concentration. The decreases in the nerve responses *fall between* the data for the reductions of the responses to moderat and high NA concentrations.

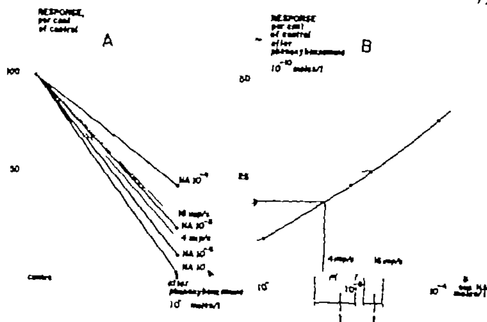


Fig. 1 A. Effect of partial α-blockade on rat portal vein responses to NA injected into the bath and to sympathetic postganglionic fibre stimulation.
Fig. 1 B. Calculation of transmitter concentrations by interpolation. Mean ± S.E.M. at 4 and 16 imp/sec from 15 experiments are indicated.

It is suggested that the relati e reduction of an adrenergic response by a partial α-receptor blockade mainly is dependent on the concentration of NA at the receptors, irrespectively of whether th agent has been released at nerve stimulation or administered *via* external routes. It should then be possible to estimate approximately the transmitter concentration t the neuro-muscular junctions by comparing the reductions of the responses. In Fig. 1 B the percent responses remaining after the partial α-blockade shown A are plotted against log concentrations of NA. The corresponding concentrations t nerve timulation at 4 and 16 imp/sec ha e then been interpolated.

The transmitter concentrations (mean ± S E M) thus obtained in 15 experiments are (8 ± 4 10 at 4 imp/sec and (2 ± 0 5 10 at 16 imp/sec. These alues are in accordance with findings, arri ed at by ther methods, recently reported. (Folkow Häggendal and Lisander 1967)

Ref rences

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D 6

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Osmotic effects on isolated vascular smooth muscle as reflected in the distribution of water, urea-^{14}C and sucrose-^{14}C

By

A. Arvill, B. Johansson and O. Jonsson

A previous study (Johansson and Jonsson, 1968) indicated that electrical and mechanical activity in the smooth muscle of the isolated rat portal vein is correlated to the volume of the smooth muscle cells. Shrinkage of the cells by increased external osmolarity decreased the activity and osmotic procedures that would make the cells swell caused excitation. The time course of the responses to increased osmolarity produced by adding sucrose and urea, respectively could be understood on the basis of the above hypothesis if it were assumed that the smooth muscle cell membranes are easily permeable to urea but relatively impermeable to sucrose. The present study has shown that the ultimate distribution of urea-^{14}C in the isolated portal vein agrees satisfactorily with total tissue water which is approximately 79 per cent of the muscle mass when determined by drying to constant weight. The transient inhibition of muscle activity obtained in a Krebs solution made hyperosmotic by adding urea to the solution and the excitation seen on return to normal solution after variable periods of exposure are entirely consistent with the rapid libration of urea-^{14}C in the portal vein preparation. The magnitude of the excitation seen on return to normal solution depends on the duration of the preceding exposure to hyperosmotic urea Krebs. This response is maximal after exposures of 5 min or more which agrees well with the fact that the uptake of urea-^{14}C reaches its plateau in about 5 min.

The distribution of sucrose-^{14}C corresponds to approximately 32 per cent of total tissue within one min and then slowly increases to approximately 48 per cent in one hour. This is consistent with a prompt and sustained osmotic effect of hyperosmotic sucrose-Krebs on the smooth muscle cells as suggested on the basis of the inhibition of activity (Johansson and Jonsson, 1968).

By combining determinations of extracellular space (sucrose-^{14}C) with standardized weighing of the muscle in isosmotic and hyperosmotic (addition of sucrose) environment it has been shown that the vascular smooth muscle cells shrink approximately in proportion to the increase in extracellular osmolarity thus behaving essentially as osmotic cells. The above results support the previous suggestion that the vascular smooth muscle activity is dependent on the cell volume.

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D 7

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Preparation of aqueous samples for liquid scintillation counting with the aid of freeze-drying

By

B. Waldeck

Water is by far the most commonly used solvent in analytical chemistry In a liquid scintillation system water acts as a quencher With the use of a suitable solvent system *e.g.* dioxane-naphtalene it is possible to introduce up to 20 per cent water Acid solutions have to be neutralized before counting In this laboratory there was a need for measurement of ^{3}H and ^{14}C-labelled monoamines in 5—20 ml hydrochloric acid eluates from ion-exchange columns. The problem was solved by freeze-drying the samples directly in the counting vials

Fig 1 Freeze-dr[illegible] eq[illegible] pment for preparation of aqueous samples in liquid [illegible] cou[illegible]

A Vacuum chamber [illegible] lose[illegible] [illegible] perspex lid. B Vacuum[illegible] tra[illegible] [illegible] U-shaped freezing [illegible] D Valves for control of vacuum and the [illegible] freezers E Freezing machine F Vacuum pump

The vacuum system has been developed and built in the laborato[illegible] workshop [illegible] co-operation [illegible] M [illegible] Andersson. The freeze-unit [illegible] manufactured by [illegible] Barle [illegible] Denmark

The freeze-drying equipment is shown in Fig 1 The samples, solidly frozen in counting vials, are placed on a heated tray of aluminium with bores for 33 samples. The tray with the samples is then put into a vacuum chamber and connected to electrical power supply and temperature control. In the bottom of the chamber there is an U-shaped tube of acid-resistent steel cooled to -80 C by methanol pumped from a tank situated behind the vacuum chambers. The methanol is cooled by a two-stage freezing machine with a net capacity at the working temperature of about 300 kcal/hr The vacuum chamber is closed by a perspex lid and the pressure brought down to 10^{-2} mm Hg by a two-stage gas-ballasted vacuum pump (Edward ED 250) The sample tray is heated electrically with about 30 watts. Under these conditions 1 ml/hr of N HCl will evaporate from each vial. The acid will be almost quantitatively recovered from the freezing element. Working with N or stronger hydrochloric acid it is essential to keep the temperature of the freezing element low. Above $-60°$ the samples will melt and splash, resulting in serious contamination

After drying, 5 ml of a mixture of 10 parts of toluene, containing 3 g PPO and 0.3 g POPOP per litre and 1 part ethanol, containing 1 % conc. HCl, are added to each vial. The vials have to be properly shaken prior to counting in a liquid scintillation spectrometer The counting efficiency for ^{3}H is about 30 % and for ^{14}C about 80 %

The method may be employed also for other aqueous solutions. However a necessary condition is that the labelled compound to be measured is non-volatile at the pressure and temperature in question.

COMMUNICATIONS

C 1

Department of Physiology University of Lund, Sweden

Regional hyperosmolality in relation to exercise hyperemia

By

S D Gray J Lindvall and S. Mellander

In a recent publication (Mellander *et al.* 1967) muscle exercise was shown to in crease regional osmolality up to 40 mOsm/kg, above resting level. Intra-arterial infusion of hypertonic glucose or xylose solutions into resting muscle evoked a dilatation of the resistance vessels sometimes approaching that seen in exercise. Other regional vascular effects mimicked those during work. Hyperosmolality inhibited vascular smooth muscle in vitro. From these results it was postulated that regional hyperosmolality could be a factor partly responsible for exercise hyperemia.

This hypothesis was further examined in the present study Graded experimental regional hyperosmolality in cat skeletal muscle, up to 40 mOsm/kg above control, elicited proportional dilatations of the resistance vessels to yield maximal flow rates of about 30 ml/min × 100 g tissue at normal pressure head Capillary filtration coefficient (CFC) reflecting precapillary sphincter tone, rose to high levels indicating pronounced sphincter relaxation The capacitance vessels, however showed no active dilatation as evidenced by following changes in regional blood volume (plethysmographic and Cr^{51} isotope techniques)

Hypertonicity about 40 mOsm/kg above control modified the normal vascular response to prolonged sympathetic stimulation significantly There was an initial transient resistance response of about the same magnitude as during control stimulation However in contrast to the maintained control response flow increased within 1 to 2 min of continued stimulation giving steady state resistance response averaging 25 of the control value The active constriction of capacitance vessels was, however well maintained, the initial response being 95 and the steady state response 8_ of control CFC in the steady state flow period, in hypertonic and control situations was not significantly altered by the stimulation.

These data show that hypertonic infusion influences the consecutive vascular sections in skeletal muscle and modifies the vascular response to sympathetic stimulation in much the same way as is known to occur in muscle exercise (Kjellmer 1965)

Other experiments indicate that interstitial deposits of Xe^{133} are cleared at faster rate when dissolved in hypertonic than in isotonic medium. Expe

human forearm (Lundvall, Mellander Westling and White to be published) show considerable increases of regional osmolality during rhythmic exercise. Intra-arterial hypertonic infusion to the forearm produced significant increases of blood flow thus confirming the animal experiments.

The present data lend further support to the hypothesis that regional hyperosmolality can be one factor in exercise hyperemia.

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C 2

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Capillary diffusion capacity in human skeletal muscle studied by local injection of radioactive tracers

By

N A Lassen and J Trap-Jensen

The clearance rate of locally injected radioactive extracellular ions were studied in hyperemic human skeletal muscle (m. tibialis ant.) ^{24}Na ^{131}I ^{51}Cr-ethylene-diamine tetra acetate (a monovalent chelate anion) and ^{57}cobolt-cyanocobalamine (containing 0 1 mg unlabelled B_{12} per injection) were employed as tracers. The local blood flow was estimated by the ^{133}Xenon clearance method. With all the four extracellular tracers the clearance rate was much smaller than could be explained on the basis of blood flow limitation in accordance with Renkin and Crone's concepts a diffusion barrier apparently limits the tissue to-blood exchange of small hydrophilic molecules in hyperemic skeletal muscle.

The Capillary Diffusion Capacity (PS of Renkin) was calculated in terms of the unidirectional flux of the tracers CDC is the number of ml of extracellular fluid rinsed completely of tracer in the (imaginary) situation of infinitely high capillary blood flow i.e. when no back diffusion of tracer occurs.

In 6 normal men the following average values were obtained CDC_{Na} 5.1 $CDC_I = 5.2$ $CDC_{EDTA} = 3.4$ $CDC_B = 1.7$ ml of extracellular fluid per 100 grams and per minute (= mol/ml/100 g/min) The increased CDC found in patients with long term diabetes was also discussed (Trap-Jensen et al. 1967 1968) It is concluded that the local clearance method allows one to study the capillary permeability in human skeletal muscle with relative ease and minimal risk or discomfort to the patient.

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C 3

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Patterns of convergence at different levels of the Group I afferent pathway to the cat cerebral cortex

By

I Rosén

The convergence of excitation in the group I pathway to the cortex has been studied in animals anaesthetized with Nembutal by recording extracellular potentials from the two relay nuclei, the cuneate nucleus and the n. ventralis posterolateralis. Relay cells were identified by antidromic stimulation of the medial lemniscus and the cerebral projection areas, respectively

Stimulation of nerves innervating muscle groups acting at various forelimb joints (Fig. 1 A) has shown that the majority of the cuneate cells are activated from one nerve only (Rosén 1967) The pattern is similar at the thalamic relay of the pathway Previous studies on the cerebral cortex indicated a much more extensive convergence (Oscarsson, Rosén and Sulg 1966)

In experiments designed to study the convergence from synergistic muscles the pattern is different (Fig. 1 B and C) The thalamic cells show a significantly higher degree of convergence than the cuneate cells.

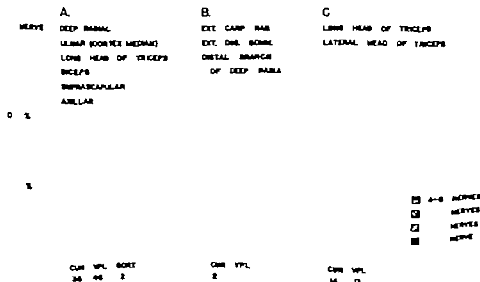


Fig 1 Convergence of group I excitation to individual cells at various levels of the group I pathway to the cerebral cortex. The forelimb nerves stimulated in the different types of preparations are indicated above each column. Numbers of cells investigated in each case are indicated below
Abbreviations CU[illegible] ate nucleus; VPL, n. ventralis posterolateralis; CORT

It is concluded that among afferents from muscles acting at different joints and from antagonistic muscles convergence appears to take place mainly in the cortex. In contrast extensive convergence from synergistic muscles is already established at the thalamic level

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C 4

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Cortical projections of Group I muscle afferents from the hindlimb

By

S. LANDGREN and H. SILFVENIUS

Projections of large muscle spindle afferents from the cat's contralateral forelimb to the cerebral cortex of the postsigmoid gyrus were described by Oscarsson and Rosén (1963). Many investigators have failed to observe projections from the hindlimb. As the movements of the cat's forelimb are more complex than those of the hindlimb a higher degree of forelimb corticalization was not unexpected. When studying projections of Group I muscle afferents to the anterior suprasylvian sulcus SII Landgren, Silfvenius and Wolsk (1967) did, however find Group I responses from the hindlimb. The Group I projections from the hindlimb to the postsigmoid gyrus were therefore reinvestigated in cats anaesthetised with chloralose.

Group I afferents from the contralateral m. quadriceps (Q) m. biceps posterior—semitendinosus (PBST) and m. gastrocnemius—soleus (G) do project to two different loci, one on the dorsal and one on the medial surface of the hemisphere. The location is shown in Fig. 1. Large muscle spindle (Ia) and Golgi tendon organ afferents (Ib) both contributed to the cortical response. The evoked potentials ap-



Fig. 1 Cortical surface potentials (CAT-average of 50) from medial Group I locus. Time: 5 msec. Voltage scale 80 µV. Dorsal root records of the afferent volley (PBST) on lower beam. The diagram shows location of Group I projection from the forelimb (stippled) and from hindlimb (hatched). SCr = Sulcus cruciatus. Cc = corpus callosum.

peared just above the threshold (T) for the nerve (c.f. Fig. 1) and their amplitudes grew in two steps to maxima correlated with those of the amplitudes of the I ... Ib dorsal root volleys. The latencies of the cortical potentials were short (8 ... 11 msec) and similar in the two loci.

The cortical potentials evoked by Group I afferents remained after ... the dorsal columns at C1 and C3 but disappeared after a superfi... section in the dorsolateral fascicle at the level of upper C1 on the side of th... Cerebellectomy did not influence the cortical responses. The ... hindlimb thus differs from that of the forelimb, which travels in th... The hindlimb path ascends with the dorsal spinocerebellar tract ... brainstem collaterals of this path.

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C 5

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Nervous control of transcapillary transport in canine mandibular tissues

By

L. EDWALL

The existence of nervous vasomotor control in mammal... reported almost a century ago but very little is known ... significance.

The aim of the present work was to investigate the transcapillary transport of ... substances in different mandibular tissues of dental ... including gingiva, dental pulp and periodontal membrane (including periodontal ... have also investigated the nervous vasomotor control in these ...

Experiments were performed on nembutal-anesthetized ... pyrine and Xe^{133} both dissolved in isotonic sal... Ten to 50 µl were injected during 4–6 min ... appearance was followed close to the final backgr... and scaler. The net pulse rates were plotted ... ner. In order to check the results obtained ... analyzed by the least square method. The ... lated at supramaximal voltage and impulse dur... 0.1 and 15 imp/sec.

The resulting curves indicate that the disappe... 60 min was multi-exponential and was followed by ...

mathematical analysis confirmed the graphically obtained results and showed three exponential functions and a constant background. One of these three rates dominated the other two were of short duration. All estimations were performed during the mono-exponential phase. The disappearance constant (k value) was significantly lower in the periodontal membrane than in the mucosa, dental pulp and gingiva.

The stimulation responses were expressed as the percentage reduction of the disappearance constant in relation to the mean of the disappearance constants before and after the stimulation. With this variable as dependent, regression analyses were performed. Among the factors studied the stimulation frequency was found to be the most important. In all tissues studied, stimulation-frequencies of up to 3 imp/sec induced increasing responses. Following higher stimulation frequencies, the disappearance of the tracers was almost completely inhibited in the periodontal membrane. This was not the case in mucosa and gingiva. In the stimulation range up to 2 imp/sec the observations indicate that the relation between stimulation frequency and response is of a similar nature in the various tissues.

C 6

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Effect of a histaminase inhibitor on the pressor response to acute alveolar hypoxia

By

A. Hauge

It has been demonstrated in isolated perfused rat lungs that local stores of histamine within the lungs are necessary for the elicitation of pressor responses to acute alveolar hypoxia (Hauge and Melmon 1968) Depletion of such stores, using compound 48/80, to less than 10 per cent of normal lung tissue content completely abolished the responses.

The major pathway for inactivation of histamine in rat lungs is by its oxidative deamination to β-imidazoleacetaldehyde catalyzed by the enzyme (or group of enzymes) diamine oxidase histaminase (Buffoni 1966) The purpose of the present work has been to investigate the effect of the most active histaminase inhibitor known, aminoguanidine (Schuler 1952, Buffoni 1966) on the pressor response to acute alveolar hypoxia. The drug potentiates histamine-induced contractions of guinea-pig ileum (*Arunlakshana et al.* 1954)

Isolated rat lungs were perfused with constant volume, pulsatile blood flow at 37 C and ventilated with positive pressure inflations (Hauge 1968) Inflow pressure (P_{PA}) and effluent blood oxygen tension ($P_{\bar{V}}$) were recorded. E ery 10th min 2—4 min periods (constant in each experiment) of entilation with a 2 % O_2

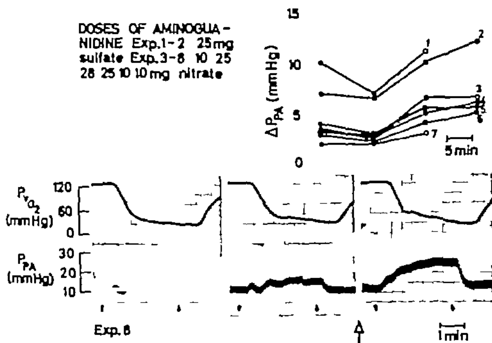


Fig. 1 Constant olume perfusion of isolated rat lungs. *TOP* △ P Inflow pressure increases to 2—4 min periods of ventilation hypoxia before ( ) and after ( ) addition of aminoguanidine. *BOTTOM* Effluent blood oxygen tension (P $_{O_2}$) and inflow pressure tracings (P A) from exp. 8. Small closed arrows indicate onset and offset of ventilation hypoxia. Open arrow indicates addition of aminoguanidine.

gas mixtu were applied, the control gas being 21 O₂. When stable or falling pressor responses to such standardized hypoxic tests were seen, aminoguanidine nitrate or sulfate were added to the blood reservoir between two tests. The results and concentrations of drug for all the eight experiments are summarized in Fig. 1. In all the lungs aminoguanidine potentiated the pressor response to acut al eolar hypoxia. by factor arying from 1.5—2.3

Although nspecific drug-effects cannot be ruled out the findings strengthen the concept that histamine is in ol ed in this response to hypoxia

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C 7

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Close correlation between single cell discharges and EEG activity in an isolated cortex preparation

By

A. Gidlöf and U. Söderberg

Single cell discharges and EEG have been recorded simultaneously from large isolated cortical slabs in cats with brain stem lesions after recovery from anaesthesia, extending the technique of Jöbsis and Söderberg (1961). Slabs prepared in this way show a remarkably stable pattern of spontaneous activity (revealed as EEG bursts separated by electric silence) that can be experimentally altered and quantitatively described (Gidlöf and Söderberg 1964).

In this work, the relation of single unit activity to EEG was studied. The majority of active neurones discharged with grouped spikes only during the bursts of EEG activity that could be recorded from the whole surface of the slab. All these neurones were silent during the isoelectric EEG. When firing occurred during EEG silence it always had the character of an injury discharge. The electrical or mechanical threshold for inducing a burst in a silent period prior to its expected spontaneous occurrence was low despite regularity in the spontaneous appearance. Minute movements of the recording glass microcapillary stimulating probably only a few neurones often sufficed to induce a burst indistinguishable from those occurring spontaneously. Thus, spontaneous activity in the isolated cortex preparation does not demonstrate the existence of a true cortical pace maker (*cf.* Bremer 1958). The slab still offers opportunities in cortical excitability studies.

Since the relation between EEG and single-unit discharges was independent of the position of the microelectrode, it seems reasonable to assume that most cells are active only during EEG bursts. Rarely records were obtained that showed a transient rise of firing rate just after the cessation of the EEG burst, like some kind of off-element. For this reason and from the findings that the average firing rate within the grouped unitary discharge was constant throughout each EEG burst and that each burst ended abruptly in the whole cortex, we suggest that activity in the cortical slab is regulated by the interaction of neurones and not, for example, by cyclic metabolic changes.

Earlier work on the slab concerned the relation of cortical blood flow to activity and to net exchange of glucose and amino acids with the finding of a rapid rise in blood flow upon each EEG burst (Jöbsis and Söderberg, 1961; Söderberg 1962; Heinichen and Söderberg, 1968). The present results demonstrate that cellular activity with discharges of action potentials is necessary for the appearance of the EEG in the isolated cortex (which, of course, differs from the intact cortex by the ab-

sence of subcortical influences) and that the rise in cortical blood flow and metabolism a few seconds after the beginning of each burst of electrical activity is a direct result of that activity

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C 8

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Multiple thalamic sources of spontaneous rhythmic activity

By

P Andersen, G F Henriksen and K. Junge

According to the Morison/Jasper theory (Dempsey and Morison 1942, Jasper 1949) the midline and intralaminar thalamic nuclei serve as a general pacemaker for spontaneous 10/sec thalamic and cortical activity However since normal cortical spindle activity can be recorded after complete bilateral removal of the midline and intralaminar nuclei, this theory is no longer tenable (Andersen. Andersson and Lømo 1967a)

These results promted an investigation into the possibility of localizing a single pacemaker elsewhere in th thalamic complex or whether multiple pacemakers had to be proposed. Logically any pacemaker must ha e a greater tendency to start rhythmic activity and a higher internal spindle freq ency than driven areas

We have used four micro-electrodes for simultaneous recording within different thalamic nuclei. The electrodes were carried in pairs in two micromanipulators and placed from 0 8 to 5 mm apart in a frontal, horizontal or sagittal plane. The spike activ ty at all activ electrode sites were recorded simultaneously on tape Spindle sequence was recorded on moving film. The interval histogram from each electrode position was calculated by help of a data retrieval computer

The most triking result was the ariability of the spindl acti ity both w th position nd time Therefore, the intra-thalamic rhythmic activ ty had four-dimensional pattern. Great changes in rhythmic pattern was seen by d ancing the electrode less than 1 mm. Thus, the results prevent any description of the distribution of hythmic activity after a rigid pattern. Typical spindle activ ty could be recorded from all regions of th thalamus. The intraspindl burst frequency increased w th increasing recording depths and bursts were more commonly seen in the lateral than i the medial half of the thalamic complex.

When spindles occurred nearly synchronously in 3 or 4 electrodes, there was a tendency for an active electrode site to remain the "leading point" (showing spindles first) for several spindles in succession, to be relieved by another electrode after some cycles. The more lateral and deeply located recording points had a greater tendency to serve as "leading points"

In conclusion, within the thalamic complex no place was found that could be called a general pacemaker area. The results support the facultative pacemaker theory of multiple sources of rhythmic activity provided the local conditions of excitation are at an optimal level (Andersen, Andersson and Lømo 1967b)

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C 9

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Renal excretion and volume of distribution of polyethylene glycol (PEG) in the rat and the dog

By

F BERGLUND

In the rat the renal clearance of inulin averaged 80 % of the clearance of PEG 1000 (Berglund 1965) indicating a relatively small pore size of the glomerular membrane. The renal clearances of PEG of various molecular weights were therefore measured by a constant infusion technique. Mannitol was used as reference substance Restriction of filtration of PEG appeared at a molecular weight below 4000 or an Einstein-Stokes radius of 17 Å (Fig. 1) In the dog restriction to glomerular filtration of PEG seems to occur at a molecular weight 2000 units higher than in the rat, or at approximatel 21 Å (Shaffer et al. 1949) This agrees well with the value of 20 Å obtained by Wallenius (1954) with dextran.

To test whether the difference between the two species also applied to peripheral capillaries, the volume of distribution of PEG was measured. PEG 6000 showed volume of distribution (14—17) approximating extracellular fluid volume and PEG 9000 and 20000 (5—8) approximated plasma volume in both species. These data do not indicate n marked species diff rence in permeabil ty of peripheral capillaries.

At low molecular weights of PEG volume of distribution approached the volume of total body water in both species, indicating intracellular distribution. This occurred with much larger molecules in th rat than in the dog From the rate of the

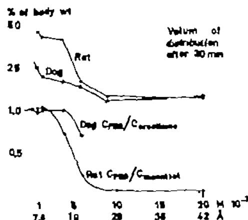


Fig. 1 Volume of distribution nd renal clearance ratios of polyethylene glycols. Molecular eights and Einstein-Stokes radii on abscissa.

osmotic to the diffusion flow of water into the cells, Villegas t al. (1955) calculated an effectiv pore radius in dog erythrocytes of 7 4 Å, corresponding to PEG of molecular weight 645. In the rat cell membrane the "effecti e pore radius" seems to be around 17 Å.

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C 10

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The effect of aldosterone and corticosterone on the (Na⁺ + K)-activated ATP hydrolyzing enzyme system in kidneys of adrenalectomized rats

By

P L. JORGENSEN

The gradual decrease in amount of the (Na +K) ATPase in kidney after adrenalectomy in rats corresponds with the rat of change in plasma N and K This suggests an involvement of this enzyme in the regulation of the renal excretion of cations.

A high sodium intake can postpone and partly *prevent the decrease in acti ty* after drenalectomy It is therefore doubtful whether the adrenal steroids can ha e a direct influence on activity of the enzyme. To get more direct information on this, the effect of the steroids that dominat the secretion of the rat adrenal cortex ha e been studied.

As previously described (Jorgensen 1968) the quantitative analysis of this enzyme in the subcellular fractions from kidney involves demasking of a considerable latent activity. Steroid treatment of adrenalectomized rats supported with saline were started 7 to 9 days after adrenalectomy when the amount of (Na + K) ATPase in kidney is 35—40 % below the normal level.

After injections at 5 hr intervals of 5 μg d,l-aldosterone (per 100 g rat) plus corticosterone in doses near the reported secretion rate of the adrenals, an increase in activity was apparent after 8 hrs. After 42 hrs the activity was about 10 % below that of normal rats, and significantly higher than after treatment with corticosterone alone.

The effect of corticosterone alone was slow in onset and after 64 hrs the activity was about 25 % below the normal level. Aldosterone alone also increased activity of the enzyme, but to a lower extent than in combination with corticosterone.

The changes in plasma Na and K always preceded the additive effects of the two steroids on the amount of the (Na + K) ATPase in kidney. It seems however to be a condition for restoration of the enzyme to normal levels, that the steroid treatment leads to a change towards normal in the imbalance of Na and K after adrenalectomy. These results, and the influence of the supply of sodium to adrenalectomized rats on the enzyme, suggests an influence of Na or of the Na/K ratio on the rate of synthesis or destruction of important parts of this enzyme system rather than a primary regulation by the adrenal steroids.

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C 11

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Simultaneous recording of aortic baroreceptor activity and aortic diameter

B

H. Aar and S. Leraand

In studies on the carotid sinus Landgren (1952) and Peterson (1960, 1966) found that changes in baroreceptor act[ivity] during administration of catecholamines were caused by changes in distensibility of the carotid sinus and not by changes in sensit[iv]ity of the stretch receptors. Th[e] relationship between receptor activity and vascular diameter in the aortic arch has not been examined.

In preliminary studies in rabbits it was often observed that the relationship between aortic nerve activity and aortic blood pressure was different d[ur]ing bleeding and reinfusion. The present investigation was undertaken to examine whether this was caused by altered aortic distension or by a change in receptor sensitivity to stretch.

Rabbits were anesthetized with chloralose and urethan. The aortic diameter was measured by means of two piezo-electric crystals implanted on the ascending aorta one week prior to nerve recording. The crystals, measuring about 1×? mm, had a resonant frequency of about 2 Mc/sec and the transmitting crystal was energized 800 times/sec. The sensitivity of the ystem allowed readings of variations in aortic diameter to the nearest 0.01 mm. Activity in the whole left aortic nerve was quantitated by integration (Aars and Leraand 1968).

When aortic nerve activity was different at the same pressure during bleeding and reinfusion, aortic diameter at comparable pressures was found to be larger during reinfusion than during bleeding. The relationship between receptor activity and aortic diameter was equal in both situations. The difference in pressure/activity relationship was thus solely due to changes in pressure/diameter relationship during bleeding and reinfusion, and was not caused by changes in stretch sensitivity of the aortic baroreceptors.

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C 12

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Mechanisms for the action of luteinizing hormone (LH) on the carbohydrate metabolism of the prepubertal rat ovary

By

K. Ahrén, A. Bevi, L. Hamberger and L. Lundholm

The gonadotrophin LH has a pronounced timulatory effect on glucose ptake nd lactic acid product on by the solated prepubertal rat ovary (Ahrén nd Kost o 1963 Armstrong et al. 1963 Hamberger and Ahrén 1967). Two types of experiments ha now been perf ormed to analyse the cellular mechanism( ) for this ffec of LH on ovarian gly olysis.

In the first type of experiments prepubertal rat ovaries were ncubated n Kre bicarbonat buffer ontaining 3-0-methyl [14]C-D-glucose i.e. a glucose nalog which is taken p by the cells as glucose but not phosphorylated. The ac of cumula ion of this substanc in the intracellular compartment illustra here h the of nsport over the cell membrane. Addition of LH (10 µg/ml) ha w the ba ion med m increased significantly the rate of accumulation of h he analogue the intracellular water thus indicating that LH mulates be ne nsport of monosaccharides in this type of ovaries.

In the other type of experiments some of the intermediary met b

TABLE I. Effects of LH on the levels of glucose-6-P, fructose 1-6-di-P, pyruvate and ATP in whole ovaries from prepubertal rats[1]

	Conditions: without LH	Conditions: with LH	Significance of LH effect
Glucose-6-P	0.047 ± 0.004 (15)	0.047 ± 0.003 (13)	N.S.
Fructose-1-6-di-P	0.074 ± 0.006 (8)	0.134 ± 0.010 (8)	$p < 0.001$
Pyruvat	0.148 ± 0.014 (6)	0.351 ± 0.027 (4)	$p < 0.001$
ATP	1.46 ± 0.08 (13)	1.08 ± 0.07 (13)	$p < 0.001$

The ovaries were incubated for 1 hr in Krebs bicarbonate buffer containing 5.5 mM glucose. Bovine LH (NIH LH-B4) was present in a concentration of 100 µg/ml. The values are calculated and expressed as µmole/g wet tissue weight, and are given as mean ± S.E. Number of observations in parenthesis.

sequence of reactions leading from glucose to lactic acid have been measured by enzymatic methods. It can be seen from Table I that LH in vit markedly increased the levels of fructose 1-6-diphosphate and pyruvate, while there was no difference in the tissue level of glucose-6-phosphate between ovaries incubated with and without the hormone. A possible interpretation of this result is that LH, directly or indirectly increases the phosphofructokinase activity thus leading to an increase of its product, fructose 1-6-diphosphate.

It can also be seen from Table I that LH decreased the concentration of ATP in the ovary under the same conditions as it increased the glycolysis. An attractive pothesis is that this decreased ATP level is the factor which leads to an increased brane transport of glucose and an increased phosphofructokinase activity after ulation by LH.

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C 13

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Plasma testosterone levels and urinary LH excretion following surgical trauma in man

By

H. CARSTENSEN, N. TERNER, L. THORÉN and L. WIDE

The level of testosterone in peripheral male plasma may be regulated by a dual closed loop control consisting of receptors in hypothalamus sensing both the level of testosterone and of LH in blood reaching them either by the systemic circulation or

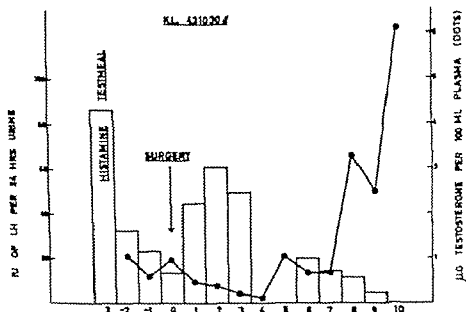


Fig. 1 Plasma testosterone levels (dots) and LH excretion alues (squares) In 24 ear old man subjected to histamine test-meal and to entricular resection.

in the case of LH, by portal essels that carry blood toward the periventricular region of the hypothalamus. As in the negative feedback control of the corticosteroid levels, both load and variable set point lements may influence the testosterone level (cf. Yates and Urquhart 1962). Surgical t auma is believed to increase adrenocortical secretion by elevating the set point. Circulating corticosteroid levels increase as a consequence of increased controller action and cause the wellknown catabolism of tissue proteins which lasts about 5—6 days. After 1—2 da this is followed by an anabolic phase previousl believed to be spontaneous (Moor and Ball 1952).

Plasma testosterone levels and rinary LH excretion were determined in two otherwise healthy men w th entricular ulcers before and after entricular resection. The blood samples were obtained at 9 AM w th the patient in upright position. Testoster one was determined w th a double isotope derivative method employing ^{35}S-toluene p-sulphonic anh dride and H-testosterone as marker added to the plasma (Carstensen 1967). LH was determined on samples of the 4 hr urine using a adioimmunoassay w th the use of Sephadex-coupled antibodies (Wid and Porath 1966).

There was a significant rise in LH excretion during the first th ee da s follow ng surgery while at the same time the testosterone levels in peripheral plasma decreased. The LH exc etion then ppeared to fluctuate somewhat with slightly increased levels in erchanging w th periods of normal levels for about one week.

In another case (Fig. 1) LH increased at the day of a histamine testmeal and at the day after operation. Between the testmeal and the operation the plasma testosterone was in the high-normal range and decreased at the same time as LH increased. On days 8—10 the testosterone levels rose drastically until 6 times above normal. Since this increase is accompanied by fairly low-normal LH excretion values, the reason for the increase may be a decreased metabolic clearance rate. The simultaneous suppression of the LH excretion may be caused by the increased testosterone concentration acting on the feedback elements of the hypothalamus. The decrease of LH at days 4—5 while testosterone still was low may be caused by the internal feedback through portal vessels in which case there will be no appreciable load on the system other than circulatory factors. As a result of the surgical trauma a change of set point might have occurred in this system rather than in the steroid feedback system. A resetting of the selected LH level may explain the lowering of the LH level that occurred on days 4—5.

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C 14

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Unit responses in the cochlear nucleus of the rat to bursts of pure tones and clicks

By

A. R. Møller

The aim of the present electrophysiological investigation was to find a neurophysiological explanation for certain psychoacoustic data concerning pitch perception (McClellan et al., 1965). This was accomplished by studying the impulse activity of single units in the cochlear nucleus of anesthetized rats in response to sound with well-defined spectral and temporal patterns.

On the basis of the response patterns the units investigated (total number 214) could be divided into two groups. One type of unit (comprising 170 cells) responded to continuous sound with a sustained train of discharges. Most of these units showed an abrupt rise in threshold when the tone frequency was increased above the unit's characteristic frequency (CF) in agreement with earlier findings in the cat (Rose et al., 1959).

The other type (44 cells) only responded to the onset of the continuous sound. The latter also showed a selectivity with regard to tone frequency but had a higher

threshold as compared to the first mentioned type. The responses of this sec nd type consisted of a single discharge immediately following upon the onset of th continuous sound. In contrast to the units of the first type these units showed marked selectivity to the click repetition rate when stimulated with bursts of petitive clicks. Below a certain click rate, one discharge was evoked by each lick and the firing ceased rapidly when the click rate was further increased. The frequen cy range within which each click was followed by one discharge varied from 200 t 800 pps among different units. The spectral sensitivity of these units to pure tones ranged from 5 to 20 kcps. It was also found that these units were not selective to repetition rate *per se* but rather to the duration of the silent periods between the successive sounds. These findings may thus explain why in psychoacoustic experi ments, repetiti e presentation of short sounds results in a pitch perception which is dependent on the length of the silent period between successive sounds.

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C 15

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Electrophysiological response to light in a moth species (Manduca sexta) reared from a vitamin A deficient diet

By

J Boatrius, S D Carlson G H oglund and G Struwe

Recently light and electron micr scopy tudies (1 2) demonst ated onsiderable pathology of the photoreceptor cells in moth (*M ndu a se ta*) ared f om a defined diet w thout tamin A, its prov tamin or analogs for over 20 generations The response of the compound ey of these moths to white and monoch omatic light was analysed lect ophysiologically Electroretinograms, compound recepto pot ntials, and intracellula potentials f om single eceptor units were obtained Elect retinograms f om the intact animal revealed a nearl normal response to *light lthough* in some ey the off-effect was not pronounced The excised ey (w th l d r no optic lobe) s esponded to ttenuation of white l ght of about th same order as found for th normal moth Spectral sens tivity studies with monochromatic l ght indicated tw general anges of spectral sensitivity one in the near ult a violet 3 0—410 *nm* nd second in the blue green-green (490—550 *nm*) These areas of sensitivity greement with those of normal moths of this species (3 Intrac ll la record gs f m single etinular ells revealed a resting potential which ranged from 35—60 V nd ndic ted a two-phase response to light a fast rising transient followed by maintained phase which wa sustained during th d ration f

stimulus as has also been found in other normal insect species (4). It is known that these moths do not give positive phototactic response to light. It is postulated that due to the retinular derangement and/or damage to higher order neurons, the visual information may not be processed in a normal manner.

In summary the investigations have revealed that 1. Intracellular recordings of the membrane potential and responses to light from the photoreceptors can be obtained in Lepidoptera. 2. the photoreceptor cells of the tobacco horn worm moth reared on an A vitamin deficient diet over 20 generations still retain functional electrophysiological capacities which approximate those of normal moths in spite of the conspicuous retinular deterioration that has been found by electron microscopy.

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C 16

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Effect of α- and β-receptor blocking agents on platelet aggregation and uptake of noradrenaline

By

S. BYGDEMAN

Adhesion and aggregation of platelets at the site of a vascular wall injury constitute two initial steps in thrombus formation. The aggregation *in vivo* is most likely induced by adenosine-diphosphate (ADP) released from the platelets and the vascular wall (Hovig 1963, Honour and Mitchell 1963). A similar aggregation can be induced *in vitro* by the addition of ADP and some other substances including adrenaline (A) and noradrenaline (NA) to citrated platelet rich plasma (Gaarder *et al.* 1961, O'Brien 1964). In the present study the effect of α- and β-receptor blocking agents on amine and ADP induced platelet aggregation and platelet uptake of ^{3}H NA in citrated human plasma have been studied.

Addition of A and NA to a final concentration in the plasma of 1 to 1×10^{-6} M induced a marked platelet aggregation. This aggregation was stereospecific and could be partially and completely blocked by the α-receptor blocking agent phentolamine at concentrations of 10^{-7} and $10^{-[illegible]}$ M respectively. Similar blocking effects could be obtained with $10^{-[illegible]}$ to $10^{-[illegible]}$ M concentrations of phenoxybenzamine.

The β-receptor blocking agents pronethalol and propranolol did not interfere with A and NA induced aggregation unless concentrations in the order of $10^{-[illegible]}$ to $10^{-[illegible]}$ M were used. MJ 1999 was without effect. ADP induced aggregation was not

hibited by either α or β-receptor blocking agents except at a very high concentra-
on (10^{-3} M). No inhibitory effect was observed with MJ 1999. The incorporation
of ^{3}H-NA in the platelets was only slightly affected at low concentrations of phentol-
amine and pronethalol. An marked inhibition of uptake was observed with high
concentrations (10^{-4} M) of both α- and β-receptor blocking agents. The degree of
inhibition was similar to that earlier observed in isolated sympathetic nerve granules
(Euler and Lishajko 1966).

These results indicate that A and NA induced platelet aggregation *in vitro* is
mediated through an α-receptor mechanism. The inhibition of ADP induced aggre-
gation with high concentrations of both α- and β-receptor blocking agents may be
explained by assuming an unspecific effect on the platelet membrane at this con-
centration level. The lack of correlation between inhibition of platelet uptake of
^{3}H-NA and amine induced aggregation does not speak in favour of the view that
uptake of amines is required for aggregation.

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C 17

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Hyperbaric oxygen exposure and monoamine metabolism in central and peripheral tissues of the rat

By

J. Häggendal

Several psychomotor symptoms can appear at exposure to air or oxygen of increased
pressure (see e.g. Bean 1965 and Adolfson 1965). Since there are some similarities
between these symptoms and those obtained after some psychopharmaca, and since
many of these psychopharmaca have an effect on monoamine metabolism in brain
and peripheral tissues, the monoamine levels have been studied in different tissues of
rat after exposure to oxygen at increased pressure (Häggendal 1967, 1968).

The noradrenaline (NA) levels in rat brain, spinal cord, and heart were found
to decrease with increased oxygen pressure and increased exposure time. In salivary
glands the NA levels increased during the exposure. The dopamine (DA) levels in
brain decreased but tended to increase when 1 per cent carbon dioxide was added
to the air. There was also a tendency of increased 5-hydroxytryptamine (5-HT)
levels in rat brain and spinal cord after exposure to pure oxygen. No changed cate

cholamine levels were found in brain after exposure to hyperbaric air with or without 1 per cent carbon dioxide added. In mouse brain decreased NA and 5-HT levels are found during hyperbaric oxygen exposure (Faiman and Heble 1966).

The tendency of increase of NA levels in salivary glands and 5-HT levels in brain and spinal cord may suggest increased synthesis of monoamines. However the most evident change in the monoamine levels was the decrease of the NA levels in brain, spinal cord, and heart. The effect of nerve impulses on this decrease has been preliminary studied. After transection in the mid thoracic region of the spinal cord no nerve impulses will reach the nerve terminals caudal to the lesion, since the impulses are generated from cellbodies in the lower brain stem. After 30 min of oxygen exposure at 5 ata the NA levels in the cranial part of the spinal cord were reduced to about 75 per cent of the control values and to about 60 per cent after 25 min at 7 ata. No differences were found in the caudal parts of exposured animals and of controls. The material is small, two estimations at every occasion, but appears to make it likely that the nerve impulse flow is of great importance for the decrease of the NA levels.

The difference between the changes of the NA levels in heart and salivary glands appears to be of particular interest suggesting different nerve impulse activities in the sympathetic nerves of these tissues at the oxygen exposure. Thus the same stimulus, oxygen exposure, seems to cause different reactions in sympathetic nerves in different organ systems.

Epileptic like convulsions is one characteristic sign at oxygen-intoxication at high pressure. The time of onset of the convulsions during the exposure was studied te drugs interfering with monoamine metabolism. The onset time of oxygen- ›o vulsions was shortened after reserpine and tetrabenazine pretreatment which ecrease the monoamine levels but increased after treatment with nialamide which increase the monoamine levels.

The results indicate that hyperbaric oxygen has an effect on monoaminergic neurons in central and peripheral tissues and it may be suggested that particularly the depleting effect on NA-containing neurons is of importance for some of the symptoms during hyperbaric oxygen exposure.

The work was supported by the Swedish State Medical Research Council (B68-14X 166-04).

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C 18

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The influence of prolonged severe exercise on the maximal lactate concentration in the working muscle on man

By

J. Karlsson, B. Diamant and B. Saltin

Åstrand *et al.* (1963) demonstrated that the peak blood lactate concentration after short term maximal exercise was lower when the maximal exercise was performed after several hours of heavy exercise and they suggested that prolonged exercise may cause a different kind of fatigue.

With the needle biopsy technique (Bergström 1962) muscle specimens were obtained on which the lactate concentration (Diamant *et al.* 1968) CP ATP ADP inorganic phosphate (Pi) and LDH activity (Karlsson et al. 1968) were determined. Five healthy male subjects were studied by taking muscle and blood samples at rest, at one submaximal (75 % of max V_{O_2}) and one maximal work load before and after 2—2.5 hrs cross country running.

After the prolonged exercise the blood lactate levels were found to be significantly lower after submaximal and maximal exercise (Fig. 1). The lactate concentration

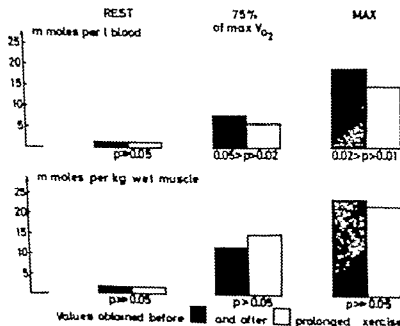


Fig. 1. Lactate concentration in blood and locally in the working muscle before and prolonged severe exercise. Student t-test is used in the statistics.

in the working muscle however was unchanged. Before the prolonged exercise the muscle tissue lactate concentrations were 2.0 12.1 and 23.8 mmoles per kg wet muscle at rest, submaximal and maximal exercise respectively as compared to 1.6, 15.0 and 21.9 mmoles after the prolonged exercise, Fig 1

The work time of the maximal work load was reduced after the prolonged exercise which means that the rate of accumulation of lactate was somewhat faster after the prolonged exercise. This could be explained by an observed lower rate of increase in oxygen uptake during the maximal work load after the prolonged exercise, and an elevated LDH activity in the exercising muscle.

During the maximal exercise after the prolonged exercise muscle ADP and P were unchanged or slightly elevated.

It is obvious that our results cannot confirm Åstrand et al.'s hypothesis that a lower "lactate production" causes the decreased work time of a maximal work load following prolonged heavy exercise, but there is no obvious explanation for the observed low blood lactate levels in the same situation.

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C 19

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Muscle temperature during submaximal exercise

By

B. Saltin and A. P. Gagge

Stolwijk and Hardy (1966) presented a model for thermoregulation in man based on input signals which were proportional to temperature deviations in the brain and the skin. Similar signals were postulated from the muscle, but as there are very few data on muscle temperature during exercise no definite conclusion can be reached.

Continuous recordings from 1—3 thermocouples in a teflon catheter placed in the quadriceps muscle were obtained in four subjects at rest and while pedalling a bicycle ergometer (50 rpm) at mean oxygen uptakes of 1.2, 2.0 and 3.2 l/min at 10° 20 and 30 C ambient temperature (T) and a relative humidity of 40 %. Rectal and skin temperatures were also monitored and sweat rate was determined by weighing the subjects.

Resting muscle temperatures (T_m) varied between 31—36° At onset of exercise it increased to a relative equilibrium within 10—12 min. After 30—60 minutes of exercise at 20° the mean T_m was 38.1 38.8 and 39.2 at the three work loads

TABLE I. Results from two subjects exercising on the same relative work load in 30 C ambient temperature.

Subject / Surface area	Max $\dot{V}_{O_2}$ l/min	Work load kpm/min	$\dot{V}_{O_2}$ l/min	$\dot{V}_{O_2}$ / max $\dot{V}_{O_2}$	T C	T_m C	T C	Sweat rate g/hr
RM 2.00 m	3.8	1050	2.60	68	38.7	39.35	29.95	690
M 2.18 m	5.2	1650	3.75	72	38.6	39.40	30.05	1100

Mean T_m was 0.6° 0.7 and 0.9° C higher than the rectal temperature (T) at 10° 20° and 30° C, respectively T_m and T during exercise both varied linearily with oxygen uptake and the best correlation was found if metabolic rate was expressed in per cent of the individual's maximal oxygen uptake. Skin temperature (T) was not correlated to T_m or oxygen uptake but varied with T There was a relation between T_m and sweating rate. For each C rise in T_m above 37.6° skin sweating rose approximately 200 g/m²×hr This may indicate that T_m has a role in thermoregulation in man. However in Tabl I are results showing that skin, rectal and muscle temperatures are almost identical during exercise t the same relative work load performed in the same environment. A possible nonthermal input directly related to the absol te magnitude of the work load may then better explain the observed differences in sweat rate.

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C 20

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Distribution of the enzymes taking part in the biosynthesis of glucuronides in the gastrointestinal tract of the rat

By

A. AITIO O HÄNNINEN and K. HARTIALA

The duodenum has the highest capacity for glucuronide formation (Hartiala et al 1964) The purpose of the present work has been to investigate formation of o-aminophenol glucuronide and the distribution of the enzymes involved, i.e. UDP glucose dehydrogenase, UDP glucuronyltransferase and β-glucuronidase in the different parts of th gastrointestinal tract.

TABLE I The rate of o-aminophenol glucuronide formation (whole wall slices) and the activities of UDPglucose dehydrogenase, UDP glucuronyltransferase and β-glucuronidase in the mucosal extracts in the various parts of the gastrointestinal tract of the rat. The value obtained for the duodenum has been denoted to 100 and the length of the small intestine to 1

	glandular stomach	duodenum	small intestine 0.1	0.3	0.5	0.7	0.9	caecum	colon
o-aminophenol glucuronide synthesis (rats)	34.0	100	67.5	39.6	22.5	17.0	14.6	15.4	30.4
UDPG dehydrogenase (8 rats)	26.8	100	60.8	45.5	26.4	28.8	38.4	127.4	70.3
UDPGA p-nitrophenol transferase (6 rats)	62.8	100	94.0	73.9	60.0	48.3	34.0	52.1	83.1
β-glucuronidase (10 rats	81.[illegible]	100	106.2	117.1	133.3	137.6	141.2	221.3	181.3

The glucuronide formation was studied by determining the rate of o-aminophenol glucuronide formation in whole wall slices (Hartiala et al 1964). UDPglucose dehydrogenase was determined by following the reduction of NAD by the soluble fraction of mucosal extracts in 0.15 M KCl. UDP glucuronyltransferase by determining the p-nitrophenol conjugation by the supernatant fraction (12 000 g 10 min) of mucosal extract in 1 digitonin solution fortified by UDPglucuronic acid, and β-glucuronidase by the liberation of phenolphthalein from its glucuronide by aqueous extracts of the whole mucosa (Hanninen 1966). Digitonin is added in order to stabilize the UDP glucuronyltransferase. When extracted into 0.15 M KCl, its activity disappears within 3 hrs.

The results obtained have been collected in Table I. The activities of UDPglucose dehydrogenase and UDP glucuronyltransferase decrease from the oral to the aboral end of the small intestine as the rate of o-aminophenol glucuronide synthesis in tissue slices, whereas the activity of β-glucuronidase increases. In the glandular stomach caecum and colon the o-aminophenol glucuronide synthesis and the three enzyme activities apparently vary independently.

These findings indicate that there are other factors than UDP glucuronyltransferase which limit the rate of glucuronide synthesis in gastrointestinal tissue slices.

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C 21

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Variations in the body surface temperature of the harp seal

By

N. A. Øritsland

It has been argued that the seal mainly achieves regulated heat loss by means of controlled heat dissipation from the limbs. The rest of the body surface has been considered to be of minor importance as an area of regulated heat loss because of the thick layer of poorly circulated blubber beneath it (Irving and Hart 1957, Hart and Irving 1959).

In the experiments concerned a harp seal was trained to walk on a treadmill (Fig. 1). Thermograms were obtained from different parts of the body surface by means of a surface temperature recording device calibrated against a black body (Bjork 1967).

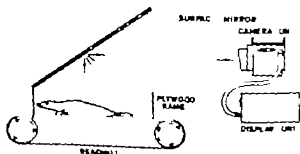
Before exercise at an ambient temperature of −13° C the surface temperature of the fore and hind flippers and the nearby body surface remained at 5—10° C. During exercise the same surface temperature rose to about 22° C and the area increased.

When the same tests were repeated at −5 and 0° C the surface areas with elevated temperature were greater than the areas at ambient −13° C.

The increase in the areas with elevated temperature was observed to start from the bases of the flippers and spread out towards the main part of the trunk. In certain cases warm spots appeared in irregular patterns on the trunk surface.

The body area which thus was assumed to take part in temperature regulation covered about 10—20% of the visible dorsal surface when the seal was at rest at −13° C. During exercise this area increased to 60—80% of the visible dorsal surface. At an air temperature of 0° C the corresponding figures were 30—40% and 70—90% respectively.

These observations suggest that regulation of both surface temperature and the area activated to increase heat dissipation may be of significance in temperature regulation of the seals.



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C 22

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Amine formation by rat mast cells in vitro

By

S. A. Slorach and B. Uvnäs

The effects of incubation temperature, time, pH, cell concentration, pyridoxal and aminoguanidine on ^{14}C histamine formation by rat mast cells *in vitro* were investigated. It was shown that disruption of the cells by physical means (sonication, freezing and thawing and hypotonic shock) or treatment with the histamine releaser ATP caused a marked reduction in histamine formation. In contrast to this, treatment of the cells with compound 48/80 caused an increase in histamine formation. After water lysis and centrifugation the histidine decarboxylase activity was found predominantly in the non-particulate fraction.

The cells were also shown to decarboxylate 5HTP and dopa *in vitro* forming 5HT and dopamine. Hydroxylation of phenylalanine, tyrosine or tryptophan could not be demonstrated. This suggests that the reason why normal rat peritoneal and mast cells contain relatively low amounts of 5HT (compared to histamine) no dopamine is the unavailability of the immediate precursors (5HTP and) and their inability to synthesise these by hydroxylation of tryptophan or tyrosine.

C 23

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Quantitative analysis of the correlation between electrical and mechanical activity in smooth muscle

By

J. Axelsson, G. Gudmundsson and B. Wahlström

A linear model of the relation between the electrical activity and the mechanical response in the portal vein (Axelsson *et al.* 1967 Johansson *et al.* 1967) is given by the convolution integral

$$Y(t) = \int_{-\infty}^{\infty} W(t-t')\, X(t')\, dt',$$

where Y is the mechanical, X the electrical response and W is a weight function. In this equation Y is the dependent and X the independent variable and W (t−t') describes the influence of X (t') on Y (t)

It is usually preferable to investigate relationships of this kind by working in the frequency domain (Craig 1964) The observations of X and Y were digitised and the Fourier transforms estimated numerically

Estimates of the Fourier transform of W were fairly consistent in the lowest frequencies. The standard deviation of the integrals of individual bursts of electrical and mechanical activity in normal solution was of the order of 10 % of the average value and the variation of the Fourier transform of W at the zero frequency appeared to be smaller

In the three consecutive bursts during exposure to noradrenaline the value of the weight function was about four times that of the normal solution. This was due to an increase in the mechanical and a decrease in the electrical response by about a factor two in each case. This supports the idea that activation to the contractile mechanism not mediated via transmembrane potential changes may play a physiological role in this muscle.

The phase difference between X and Y corresponds to a time lag of about 0.3 sec and does not seem to be much affected by noradrenaline

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C 24

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Spino-olivocerebellar pathway ascending in the dorsolateral funiculus of the spinal cord

By

B Larson, S Miller and O Oscarsson

In addition to spino-olivocerebellar pathways ascending in the ventral funiculus (VF SOCP Oscarsson and Uddenberg 1966) and dorsal funiculus DF SOCP Oscarsson 1967) further pathway has been found in the dorsolateral funiculus DLF SOCP) Precollicular decerebration was performed on cats anaesthetized initially with Nembutal The spinal cord was transected in the third cervical segment sparing only the dorsal part of the lateral funiculus on one side Typical climbing fibre responses (Eccles Ito and Szentágothai 1967) were recorded from the surface of lobules IV and V of the anterior lobe of the cerebellum in response to

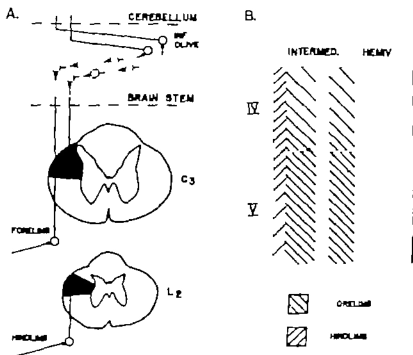


Fig. 1 A. Diagram of DLF-SOCP Location of spinal tract is show by black areas. Broken lines indicate unknow polysynaptic path.
B. Diagram of projection zones lobules IV and V of anterior cerebellar lobe Intermed pars intermedia, hemi hemivermis.

tion of cutaneous and high threshold muscle afferents in fore and hind-limb rves. The pathway projects to the ipsilateral pars intermedia and is distributed in thin sagittal bands of 0 5– 1.5 mm (Fig. 1 B) which have been confirmed by microelectrode recording from individual Purkinje cells. The latencies of the responses were 14—18 msec and 18—24 msec fore- and hind-limb nerves, respectively By means of lesions in the dorsolateral funiculus and electrical stimulation of the spinal cord, the spinal course of the pathway has been *established (Fig 1 A)* The spinal tract appears to be monosynaptically activated by primary afferents and the fastest tract fibres conduct at 60—80 m/sec. In the brain stem the course of the path is unknown, but stimulation of the tract in the pper cervical region indicates that the olive is acti ated after a delay of at least 5—7 msec. suggesting several interneurones.

The work was supported by the Swedish Medical Research Council. Project No. B68-14x-1013-03 S.M is a Best Memorial F llow in receipt of Royal Society Pergamon Travel Award.

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C 25

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Variations in the dynamic sensitivity of hind limb muscle spindles in the anesthetized cat with de-afferented lumbo-sacral cord

By

B. Appelberg and T. Jeneskog

Changes in the dynamic sensitivity of hind limb muscle spindles were demonstrated to occur spontaneously in decerebrated cats by Jansen and Matthews (1962). Also in anesthetized cats with the central nervous system intact such variations have been observed (Appelberg 1966, Appelberg and Molander 1967). In all these experiments the lumbo-sacral spinal cord could be influenced by afferent activity through some intact dorsal roots. Such reflex maintenance of dynamic fusimotor activity was recently demonstrated by Alnaes, Jansen and Rudjord (1965).

These results raise the question whether dynamic fusimotor activity is mainly brought about through spinal reflex action or if supraspinal mechanisms by themselves may contribute to a significant degree.

In the experiment illustrated in Fig. 1 the cat was under Fluothane anesthesia. The left hind limb was denervated except for the long digital flexor muscle. The dorsal root L_7 on the left side was cut, all other roots as well as the cord and the brain were intact in the beginning of the experiment.

The dynamic sensitivity of the spindle recorded from was initially rather high (A). Bilateral section of dorsal roots S to L_4 did not appreciably change the performance of the spindle during stretch (B). Bilateral lesions in the dorsal parts of the lateral funiculi also left the spindle uninfluenced (C). In this part of the cord descends a pathway through which mesencephalic stimulation causes excitation of dynamic fusimotor neurones (Appelberg and Jeneskog, unpublished observations). Not until the spinal cord was completely sectioned (D) did the dynamic sensitivity of the spindle decrease to a level identical to that of the de-efferented preparation (E).

The experiment shows that the brain may maintain dynamic fusimotor activity without the support of segmental reflexes.

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